Defining the distinct functional roles of hematopoietic and stromal antigen presenting cells in the thymus

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE
UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSPHY

Advisor: Kristin Ann Hogquist, Ph.D.

October 2020

Acknowledgements

First and foremost, I would like to thank my advisor, Dr. Kristin Hogquist. She is both an excellent scientist and an excellent person. Thank you for being an unending fountain of encouragement and kindness; for continuously demonstrating good leadership and mentorship; for supporting my interests in other career paths; and for sharing your love of research. Of the decisions I've made thus far in my life, I consider becoming your graduate student one of the best. I am incredibly grateful for the opportunity. Thank you for investing your time and effort in me.

I would like to also extend a deep and sincere thanks to the members of the joint Jamequist and Hamilton Hart labs. Along with Kris, Dr. Stephen Jameson and Dr. Sara Hamilton Hart have both been incredibly supportive and insightful. Their thoughtful leadership has shaped a joint space that is an incredible learning environment. Thank you to all my current and former lab members for their help with experiments, their insightful discussions, and their friendship: Dr. You-Jeong Lee, Dr. Roland Ruscher, Dr. Yan Xing, Dr. Hristo Georgiev, Dr. Haiguang Wang, Dr. Kristin Renkema, Dr. Kristina Burrack, Dr. Henrique Borges da Silva, Dr. Katie Block, Dr. Elise Breed, Dr. Daniel Walsh, Dr. Matt Huggins, Dr. Ryan Martinez, Oscar Salgado, Changwei Peng, Emily Truckenbrod, Kelsey Wanhainen, and Maude Ashby. Without Daniel and Elise, I probably wouldn't have even made it through prelim stress; I am incredibly grateful to have gone through graduate school with you. Our game nights with Katie are one of my

highlights during my time here. Special thanks to our lab manager, Jane Ding, who makes sure all our lab business is squared away, and sometimes gives me treats to eat.

I would like to thank all my colleagues at the Center for Immunology for their feedback and support. I am so glad to have worked in such a great environment filled with smart, generous people. Many, many thanks to my current and past members of my thesis committee, who have provided helpful feedback and support: Dr. Bryce Binstadt, Dr. Vaiva Vezys, Dr. Tim Starr, Dr. Brian Fife, and Dr. Dan Mueller. Of course, I would like to thank the MICaB program and all its members who have supported me throughout my time. Louise, Meg and the DGSs (both Dr. Jameson and Dr. Bresnahan) have been instrumental in making sure everything went smoothly. At the CFI, I would like to thank Annette Bethke and Malzona Anderson for also helping schedule my life; I couldn't have done it without you.

Finally, I would like to thank my friends (near and far!) and my family for their unrelenting support. My parents, who always checked in on both my physical and mental well-being; my brother, who I could always complain to; my partner, who I would pester relentlessly but who would still give me crunchy mints. Thank you for loving me.

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Chapter 1

Introduction

1.1 Thymus: The site of T cell development

For an effective immune response, antigens from disease-causing agents such as bacteria and viruses are recognized by the adaptive immune system. Two major components of this arm of the immune response are B and T cells, both of which have antigen receptors that respond to a vast range of antigens. These lymphocyte populations, however, develop in different tissues: for T cells, the primary lymphoid organ is the thymus. Like B cells, T cells develop from hematopoietic stem cells (HSC) in the bone marrow, and during fetal life, in the liver. Lymphoid progenitors access circulation and migrate into the thymus. There, these cells, called thymocytes, undergo differentiation and selection steps through their interactions with thymic antigen presenting cells. This process generates a mature repertoire of T cells that is functional – able to protect the organism from pathogens it may encounter, but also self-tolerant.

In all species with T cells, development occurs in the thymus. If the thymus is surgically removed (thymectomized) early in life or if patients are born with mutations that impact the development of the thymus, there is striking immunodeficiency, leading to increased susceptibility to infection[1]. Anatomically, this organ is located behind the sternum, and its distinct lobes are divided into the outer cortex and inner medulla[2]. The thymus is primarily composed of developing thymocytes, but thymic stromal cells and hematopoietic antigen presenting cells are present as well, and create unique microenvironments in these distinct regions. This compartmentalization of thymocytes provides for

distinct cues that are needed to support T cell development through the different developmental stages.

Initially, thymic precursors enter into the thymic parenchyma near the cortico-medullary junction (CMJ)[2]. As they develop, immature thymocytes migrate into and through the densely packed cortex, where they are guided by cues from surrounding cells. These cells include large, branched cortical epithelial cells (cTEC), dendritic cells (DC), and macrophages. Here, thymocytes are committed to the T cell lineage and progress through sequential differentiation steps. The thymic environment provides chemokines that later guide the thymocytes to leave the cortex into the thymic medulla. The thymic medulla has distinct thymic epithelial cells, aptly named medullary thymic epithelial cells (mTEC), as well as other antigen presenting cells. More mature thymocytes reside in the medullary region: $\alpha\beta$ T cells become MHC class I or MHC class II restricted, and become $CD8^+$ or $CD4^+$ thymocytes. These surviving thymocytes then emigrate from the thymus into the periphery as mature T cells.

Thymic stromal cells, including cTEC and mTEC, form the supporting meshwork needed for thymic structure and proper thymocyte development[3]. Foxn1 is a transcription factor essential for TEC development, and *Foxn1* deficiency leads to a thymic rudiment that cannot sustain lymphopoiesis. Notably, defects in *Foxn1* have been described in both mice and humans, with a phenotype that includes hair loss and T cell immunodeficiency[4]. In humans, this disorder is referred to as

the Pignata Guarino syndrome, and in mice, the *nude* mutation. *Nude* mice particularly shed light on the specific need for thymic stromal cells for T cell development: when bone marrow from *nude* mice is transplanted into mice with normal stromal cells, functional T cells develop, but they do not when bone marrow from normal mice is transplanted in mice with the *nude* mutation. Another human disease with a similar phenotype of athymia and T cell deficiency is DiGeorge syndrome, but these patients have deletions in chromosome 22q11, which encodes *Tbx1*. Taken together, these observations show that the thymus is the major site of T cell maturation and is necessary for functional peripheral T cells.

1.2 Positive and negative selection

Conventional $\alpha\beta$ T cells in the periphery are either $CD4^+$ helper or $CD8^+$ cytotoxic T cells, and this divergence of co-receptor expression and effector cell type is determined in the thymus. In the thymus, this decision is made after surface TCR expression: only positively selected double positive (DP) thymocytes can transition into CD4 or CD8 single positive (SP) thymocytes. The rearrangement and pairing of $\alpha\beta$ TCR genes is random, and the TCRs on DP thymocytes have specificities that react to a wide range of antigens (both self or foreign) or don't react at all. Additionally, some receptors, while potentially capable of binding to a peptide antigen, may not bind to the peptide when presented by that individual's MHC molecules. For any individual, a useful T cell is one that can recognize an antigen (such as a foreign antigen from a pathogen) presented by a self MHC

molecule. Thus, a positive selection step is needed to enrich the T cell repertoire "with MHC restricted" TCRs specific for antigens only in the context of the host's limited MHC molecules[5]. Additionally, TCRs that bind strongly to self-peptides present a risk, as these T cells may trigger autoimmunity. Therefore, a negative selection step is also required to prune autoreactive clones from the T cell repertoire. The result of these selection checkpoints is mature T cells that are MHC restricted and self-tolerant, and can be exported from the thymus into the periphery.

1.2.1 Positive selection generates a "MHC-restricted" T cell pool

In the thymic cortex, DP thymocytes audition for selection[5]. These immature cells only have a life-span of 3 to 4 days, and without engagement of the TCR, their default pathway is one of apoptosis – a form of programmed cell death called 'death by neglect'. It is estimated at anywhere between 85 to over 90% of these precursors are unable to be selected and are eliminated in this manner[5, 6]. Cells that avoid this fate undergo positive selection, in which the $\alpha\beta$ TCR binds with low to intermediate avidity to self-peptide:MHC complexes presented by cTEC, promoting cell survival. As the self-peptides presented in the thymus are displayed on the host's own MHC molecules, positive selection ensures that only self-MHC restricted DP thymocytes mature into $CD4^+$ or $CD8^+$ SP thymocytes. After undergoing positive selection in the cortex, DP thymocytes upregulate chemokine receptors such as CCR7 and migrate towards the medulla[2]. As the

thymocyte crawls through the cortico-medullary junction into the medullary region, it interacts with other thymic APC, which can drive negative selection.

1.2.2 Negative selection generates a "self-tolerant" T cell pool

Among the TCR expressed by DP thymocytes, some bind strongly to self-peptide:MHC. During the selection processes in the thymus, these autoreactive T cell clones are removed from the repertoire in a process called negative selection[5]. Apoptosis by these cells 'deletes' them from the T cell repertoire, and elimination of autoreactive TCRs via cell death is also known as clonal deletion. While the majority of negative selection occurs in cortex at the DP stage, there is also a second wave of medullary clonal deletion in more mature thymocytes [5, 7]. The deletion of self-reactive T cells clones is key in establishing central tolerance, but along with clonal deletion, there is an additional tolerance mechanism: the development of regulatory T cells (Treg). Regulatory T cells are self-reactive CD4+ T cells that express CD25 and the transcription factor FOXP3. They generally suppress immune responses, as opposed to initiating them. While they are only a small proportion of both developing CD4 SP thymocytes (~1%) and CD4+ T cells in secondary lymphoid organs (~10-15%), they play a key role on immune homeostasis[8]. This clonal deletion and Treg selection can be mediated by mTEC as well as hematopoietic APC such as dendritic cells and B cells[9].

1.2.3 Role of peptide:MHC in negative and positive selection

The model that for positive and negative selection, the signal strength of the interaction between the self-peptide:MHC and TCR differ is known as the affinity model of selection[5]. On one end, receptors that do not bind and respond to self-peptide:MHC will automatically undergo cell death after a few days — called neglect; while on the other, receptors that bind with high affinity and respond strongly undergo cell death by clonal deletion. In the middle is a window of positively selected conventional T cells with receptors that bind with weaker affinity to self-peptide:MHC. While thymocytes that have high affinity interactions with self-peptides undergo generally apoptosis and are deleted, some cell types, such as regulatory T cells and other specialized lymphocytes are more self-reactive but are able to avoid this cell fate. These agonist selected cells require other molecular factors, such as CD1d and costimulatory molecules for their selection, but there is some stochastic overlap between agonist selected cells and clonal deletion[10].

1.3 Thymic antigen presenting cells

The different antigen presenting cells in the cortex and medulla of the thymus are specialized for positive and negative selection[5]. cTEC are essential for positive selection. The crucial role that cTEC have in selection is mediated in part by their peptide processing machinery, which gives them the ability to present a largely unique peptide:MHC repertoire (also known as the peptidome)[5, 9]. In APC, peptide fragments are loaded onto MHC molecules after cytosolic proteins are degraded by proteasomes. These proteasomes have a catalytic core that

consists of three subunits: β1, β2, and β5. cTEC have the unique proteasome subunit β5t, while other APC can only express β5 or β5i. This endows cTEC with the ability to present specialized peptides produced by the thymoproteosome (specifically composed of subunits β1i, β2i, and β5t), allowing for a peptide:MHC peptidome that promotes positive selection and shapes the T cell repertoire. The thymic cortex is also home to hematopoietic APC which play a role in selection. Notably, these bone marrow derived APC, such as dendritic cells, can mediate clonal deletion, and in fact, contribute to approximately half of clonal deletion events[7]. SIRPα$^+$ DC (also known as cDC2) are found within the thymic cortex but are migratory – capable of picking up antigen from the periphery before homing to the thymus[9, 11]. Myeloid APC populations can also be found in the cortex. Thymic macrophages are a heterogenous population found throughout the thymus and are thought to be the scavengers of apoptotic cells (of which there are many, as only about 3% to 7.5% of the pre-selection T cell repertoire becomes mature T cells)[6, 12, 13, 14, 15, 16]. Thymic eosinophils and neutrophils can also infiltrate the cortex and clear apoptotic cells, but it is not their only function: eosinophils, for example, interact with DP thymocytes at the cortico-medullary junction[17, 18]. While not as well-defined as the other hematopoietic APC, myeloid APC have also been implicated in thymocyte selection and activation[17, 19]. Cortical APC clearly mediate negative selection, as cortical deletion is a proportionally larger percentage of clonal deletion[7, 20].

The thymic medulla is home to mTEC which play a crucial role in negative selection[5, 9]. These cells are specialized to express tissue restricted antigens (TRA), proteins normally produced only in one or two tissues in the body. An example of a TRA is insulin – a protein otherwise only produced in the pancreas. The thymic expression of TRA is essential to achieve self-tolerance to all proteins in the body. TRA expression in mTEC is largely dependent on a transcriptional regulator called autoimmune regulator protein (AIRE), but Fezf2, another transcription factor, may also promote TRA expression in an AIRE-independent fashion[21].

Thymic B cells also express AIRE and mediate negative selection[9]. These hematopoietic APC have an activated phenotype – higher levels of MHCII and costimulatory molecules compared to their splenic counterparts – and are found in the cortico-medullary junction and medulla. B cells are not the only hematopoietic APC in the thymic medulla; $XCR1^+$ DC (also known as cDC1) and plasmacytoid DC (pDC) are also medullary. Like $SIRP\alpha^+$ DC, pDC are a migratory population that can transport peripheral antigens to the thymus and mediate negative selection[22]. $XCR1^+$ DC are not migratory, but they also localize to the thymic medulla due to their interactions with their ligand, XCL1. XCL1 is produced by $mTEC^{hi}$ in an AIRE-dependent fashion, and the XCL1-XCR1 interaction allows accumulation of $XCR1^+$ DC around mTEC[23]. These $XCR1^+$ DC can then cross-present antigens acquired from mTEC, including TRA, and mediate negative selection.

1.4 Tolerance to temporally expressed antigens

An effective immune system is one that can respond robustly to foreign pathogens without having unwanted responses against the host's own cells and tissues. This immunological tolerance to self-antigens is enforced early during T cell development in thymus by negative selection. The phenomenon of ubiquitous and tissue restricted self-antigens being presented in the thymus is well-defined, but there are also self-antigens that are only expressed for a limited span of time. For these antigens with a temporal component, there also needs to be immunological tolerance. For example, pregnancy results in a semi-allogeneic fetus: there are non-self, paternal antigens, but also maternal (and therefore, self-) antigens. Pregnancy has a temporal component. In other words, women are not chronically pregnant. Indeed, T_{reg} are critical for immunological tolerance and successful gestation; and further, some T_{reg} found in the decidua are of thymic origin[24, 25]. These reproduction-associated antigens are expressed in the thymus in an AIRE dependent fashion, and the loss of functional AIRE is associated with pregnancy loss and reduced fertility[26, 27].

Similarly, type I interferons (IFN-I) are cytokines that are rapidly produced during viral infections and lead to a wide breadth of immunological effects. These responses are characterized by the upregulation of various interferon stimulated genes (ISG)[28]. Even without infection, type I interferon is found at low levels in the thymus, and both thymocytes and thymic dendritic cells express ISG[29, 30, 31].

Notably, during a viral infection, IFN-I production changes the self-peptide repertoire that is displayed by MHCI[32]. Given this, we hypothesize that thymic IFN-I impacts T cell tolerance. T cell tolerance could be enhanced through the presentation of interferon-induced self-peptides (an altered peptidome); but also by changes in thymic APC subsets, whether it be increased peptide presentation or overall changes in APC composition.

1.5 Specialized lymphocytes

Conventional αβ and γδ T cells are not the only cells that develop and emerge from the thymus. There are also specialized lymphocyte populations that are numerically fewer but still play important roles in host immune responses – helping maintain tissue and metabolic homeostasis. These include lipid-reactive natural killer T (NKT) cells, CD8αα+ intraepithelial lymphocytes (IEL), and mucosal-associated invariant T (MAIT) cells. Compared to conventional αβ T cells, NKT cells and IEL express TCR that are more self-reactive[10]. Thus, their development is referred to as agonist selection in the thymus and these specialized lymphocytes require distinct molecular factors for their development.

NKT cells express an αβ TCR, but rather than being peptide specific and MHC class I or II restricted, they recognize lipid antigens in the context of CD1d molecules[10, 33]. The TCR repertoire expressed by NKT cells is highly restricted, consisting of either TCR Vα14-Jα18 (mice) or Vα24-Jα18 (humans) chains paired with limited TCR Vβ chains. Due to this “invariant” TCR, these cells are

named invariant NKT (iNKT) cells. Selection of iNKT cells happens in the thymic cortex at the DP stage. However, rather than being positively selected by thymic cTEC like conventional T cells, iNKT cells are selected by other DP thymocytes presenting lipid antigens by CD1d, a non-classical MHCI-like molecule. Strong TCR signaling at this stage along with interactions between signaling lymphocyte activation molecule family (SLAMF) receptors drives agonist selection of iNKT cells. iNKT cells are specified by the expression of the transcription factor PZLF and can further differentiate into different iNKT effector subsets within the thymus. Like NKT cells, MAIT cells have a limited TCR repertoire due to restricted TCRα chain usage. These TCR generally consist of either Vα19-Jα33 (mice) or Vα7.2-Jα33 (humans) paired with limited TCR Vβ chains[34]. MAIT cells recognize metabolites of vitamin B in the context of the non-classical MHCI-like molecule MR1 and are selected for during the DP stage of thymocyte development by other DP thymocytes.

IEL are T cells that reside within the gut epithelium and include a population that express $\alpha\beta$ TCR and CD8$\alpha\alpha$ homodimers. These CD8$\alpha\alpha$+ IEL derive from agonist selected thymic precursors (IELp) and have a small repertoire size[10, 35]. During thymocyte selection, strongly self-reactive DP thymocytes undergo clonal deletion, but without CD28 co-stimulation, more cells are diverted into the IELp fate. These TCR$\alpha\beta$+ IELp can then be found within DN thymocyte population, and most are localized within the cortex in adult mice. This is a reflection of the two distinct IELp populations in the thymus: Type A IELp are PD-1^{+}, self-reactive,

and largely cortical; while a more newly defined group, Type B IELp are PD-1^{-}, non-self-reactive, and largely medullary[36]. In adults, Type A IELp are the proportionally larger population being exported from the thymus[37]. While it is known that these precursors are β2m-dependent, the relevant cortical APC needed for their development has not been identified. Given that more IELp developed in mice lacking CD28 co-stimulation, we hypothesize that this APC expresses MHC class I and does not express CD80 or CD86.

Chapter 2

Thymic selection of IEL precursors requires MHC class I on hematopoietic antigen presenting cells

2.1 Introduction

Intraepithelial lymphocytes (IEL) are resident T cells located in the gut epithelium, where they protect against harmful pathogens, while also playing a regulatory role in preserving barrier homeostasis [38, 39]. IEL are a heterogeneous population, broadly divided into conventional and unconventional subsets. Conventional IEL, otherwise known as induced IEL, are TCR$\alpha\beta^+$CD4$^+$ and TCR$\alpha\beta^+$CD8$\alpha\beta^+$ cells generated from naïve T cells that have recognized foreign antigens in the periphery. Unconventional IEL, also known as natural IELs, include TCR$\gamma\delta^+$ T cells, and TCR$\alpha\beta^+$ CD4$^-$CD8α^+CD8β^- (CD8$\alpha\alpha$) T cells. Natural IEL derive from thymic precursors, but their developmental pathway(s) remain incompletely defined. In particular, we lack an understanding of the thymic antigen presenting cells that instruct their differentiation and trafficking.

CD8αα IEL largely differentiate from thymic precursors that have received strong stimulation by self-antigens but escaped deletion, in a process termed agonist selection [39, 40, 41]. Thymic precursors to CD8$\alpha\alpha^+$ IEL (IELp) are found in the CD4$^-$ CD8$^-$ double-negative (DN) population of the thymus and are TCR$\alpha\beta^+$CD5$^+$H-2K^{b+}CD122$^+$ [36, 42, 43, 44, 45]. This precursor population is itself heterogeneous, with two major subsets that differ in both cell surface markers and T cell receptor (TCR) specificities [36, 45]. The proportionally larger IELp population – Type A IELp – are a nascent population induced by strong TCR signaling, express PD-1, and readily emigrate from the thymus. The smaller population – Type B IELp – contribute little to the emigrating IELp pool in adult mice, and are PD-1$^-$NK1.1$^+$ [36].

Nonetheless, both IELp are capable of giving rise to CD8$\alpha\alpha^+$ IEL in the gut, and recent evidence suggests Type B IELp make a larger contribution to the gut early in life, and become outcompeted by Type A IELp in adulthood [36, 37, 46]. Most IELp are restricted to classical and non-classical MHC class I (MHCI) molecules [36, 43, 44]. Strong TCR engagement of MHCI together with CD28 co-stimulation results in death of thymic precursors (negative selection), and co-stimulation-deficient mice (either *Cd28*$^{-/-}$ *or Cd80*$^{-/-}$*Cd86*$^{-/-}$) had higher numbers of thymic IELp and intestinal CD8$\alpha\alpha^+$ IEL [36, 47, 48]. Thus, avoidance of clonal deletion, called 'clonal diversion', is thought to be a major driver of IELp development. However, little is known about the thymic antigen presenting cells (APC) that lead to this diversion [36, 49]. Given the above, we predict that such APC would express MHC class I and low or no CD80/86.

While medullary thymic epithelial cells (mTEC) and thymic dendritic cells (DC) are known to drive clonal deletion in the thymus, they are not the only APC that mediate selection processes[9]. Here, we sought to determine the role of APC that support positive selection of IELp. Using bone marrow chimeras, we show that IELp require MHC class I on hematopoietic APC (hAPC). However, our data with selective depletion suggest that no single hAPC subset is responsible for IELp selection on its own. Moreover, CD80 co-stimulation alone can limit the Type A IELp fate, even without CD86. Collectively, our findings show that hAPC play a specialized role in clonal diversion, but specific thymic hAPC subsets may play functionally redundant roles.

2.2 Results

2.2.1 Hematopoietic APC are required for IELp selection in the thymus

Previous reports indicate that both CD8αα IEL in the gut and their thymic precursors of IEL are β2m-dependent, with 10 fold fewer signaled PD-1$^+$ DN thymocytes in β2m deficient models[36, 43, 44]. However, there are many different thymic APC with β2m dependent MHC molecules expressed on the cell surface, including both hAPC (such as dendritic cells, B cells, macrophages, and eosinophils) and stromal APC (thymic epithelial cells) that can determine the fate of thymocyte selection[9, 17, 50, 51]. Therefore, we first sought to differentiate between the roles of hematopoietic and stromal APC for IELp development using bone marrow chimeras. WT or β2m-deficient marrow was used to reconstitute lethally irradiated β2m-deficient or WT recipients, respectively. Using congenic markers to differentiate host- and donor-derived IELp (Fig. 2.1A; typical IELp gating without congenic markers shown in Figure 2.2), we found that a significantly lower proportion of signaled DN thymocytes developed into mature (CD122$^+$) IELp in mice with β2m-deficient marrow (Fig. 2.1B). This IELp reduction in these mice where β2m was deficient in hematopoietic cells was reflected in the reduction of both the PD-1$^+$ Type A and NK1.1$^+$ Type B IELp numbers (Fig. 2.1C).

Our finding of significantly fewer IELp with β2m-deficient hAPC was recapitulated by a reduction in CD8αα$^+$ IEL in the small intestine of these mice (Fig. 2.1D).

Taken together, our data suggest that MHC class I expression is required on hAPC to positively select developing Type A and Type B IELp, and these precursors may rely on a single or select hAPC for this activity.

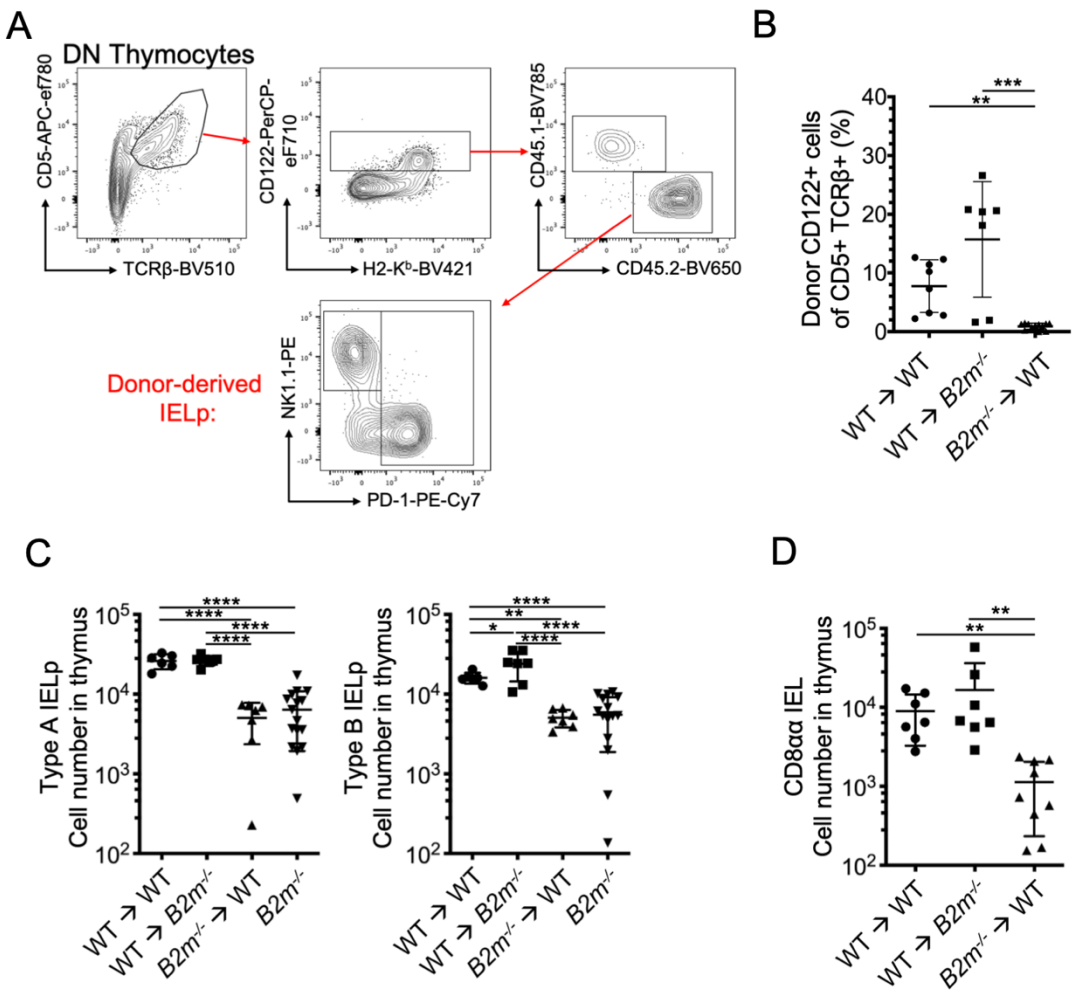

Figure 2.1. Positive selection of IELp depends on MHC class I expressed on hematopoietic APC. (A) Representative flow cytometry plots of DN thymocytes in bone marrow chimeras (WT → WT shown). Full gating for IELp shown in Fig. 2.2. Mature IELp were defined by CD122 expression alone due to the lack of H2-K^b expression in mice with β2m deficient bone marrow. Donor and host cells were differentiated using congenic markers, CD45.1 and CD45.2. **(B)** Quantified percentage of donor-derived IELp after WT or *B2m*$^{-/-}$ bone marrow transfer into lethally irradiated recipients indicated. **(C)** Absolute number of PD-1$^+$Type A IELp (left) and NK1.1$^+$ Type B IELp (right) after WT or *B2m*$^{-/-}$ bone marrow transfer into lethally irradiated recipients indicated. **(D)** Absolute number of small intestine CD8αα IEL in the indicated bone marrow chimeras. Each symbol in (B-D)

represents an individual mouse. Data are pooled from at least three independent experiments. Error bars show mean ± SD. *p≤0.05, **p≤0.01, ***p≤0.001, ****p ≤ 0.0001, ANOVA with multiple comparisons.

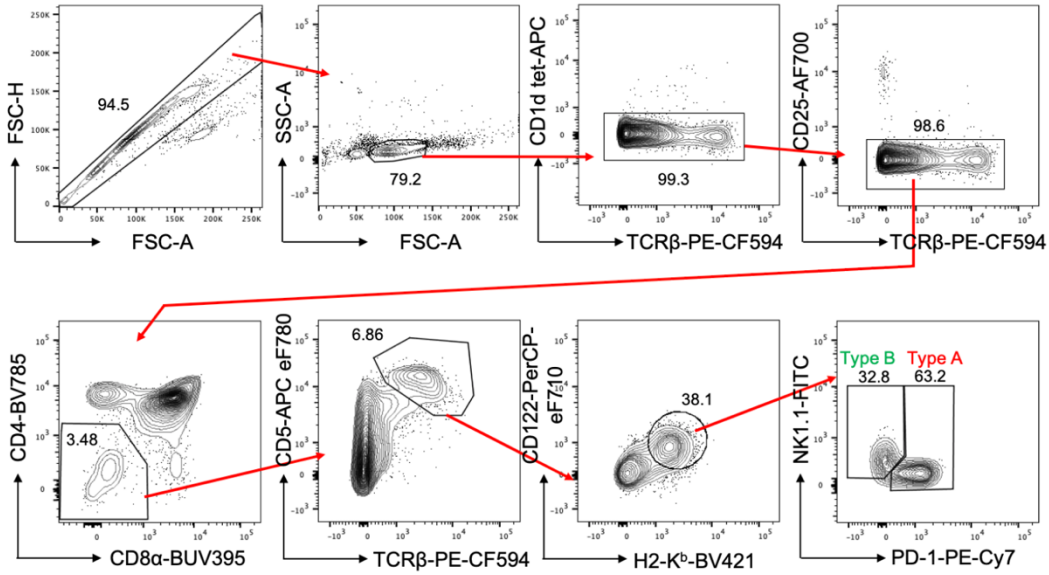

Figure 2.2. Gating for IELp. Representative flow cytometry gating strategy for type A and type B IELp. Signaled DN thymocytes identified by the expression of CD5 and TCRβ, after excluding PBS57-CD1d tetramer$^+$, CD25$^+$, CD4$^+$, and CD8$^+$. Mature IELp were identified by CD122$^+$H-2K^{b+}. Type A IELp identified as PD-1$^+$ and type B as NK1.1$^+$. Numbers adjacent to the outlined areas indicate the percentage of cells in each.

2.2.2 CD28 co-stimulation drives cells away from the Type A IELp fate

Most T cell precursors that interact strongly via their TCR with self-peptide:MHC undergo apoptosis. However, some cells (including IELp) can escape deletion, and these high-affinity TCR interactions lead to other cell fates in a process termed “agonist selection”[10, 49]. These agonist-selected populations include T regulatory (Treg) cells, which require CD28 co-stimulation by CD80 and/or CD86 for their development and function[52, 53]. In contrast, IELp are agonist-selected cells that are positively selected in the absence of CD28 co-stimulation[36, 48]. For the process of clonal deletion, the ligands for CD28 –CD80 (B7-1) and CD86 (B7-2) – are not equivalently expressed, and several APC throughout the thymus express CD80 alone[7]. Given these patterns, we evaluated the role of costimulatory molecules in IELp development in CD86 knockout (KO) and CD80/CD86 double KO mice. Consistent with previous reports using CD28 mice, the number of Type A IELp increased in the CD80/86 double KO mice[36, 48] (Fig. 2.3A). Further, the double KO rescued IELp and CD8$\alpha\alpha$ IEL numbers more effectively than CD86 deficiency alone, suggesting both CD80 and CD86 restrain the Type A IELp fate in the thymus(Fig. 2.3A, 2.3B).

In agreement with our previous study indicating that Type B IELp are not rescued in CD28 deficient mice, numbers of thymic Type B IELp were not increased in CD80/86 double KO mice compared to WT and, in fact, trended in the opposite direction[36] (Fig. 2.3A). Patterns for CD8$\alpha\alpha$ IEL numbers followed Type A rather

than Type B IELp numbers in the double KO mice, which may be a reflection of the Type A IELp population being the proportionally larger IELp subset regardless of co-stimulation status (Fig. 2.3C).

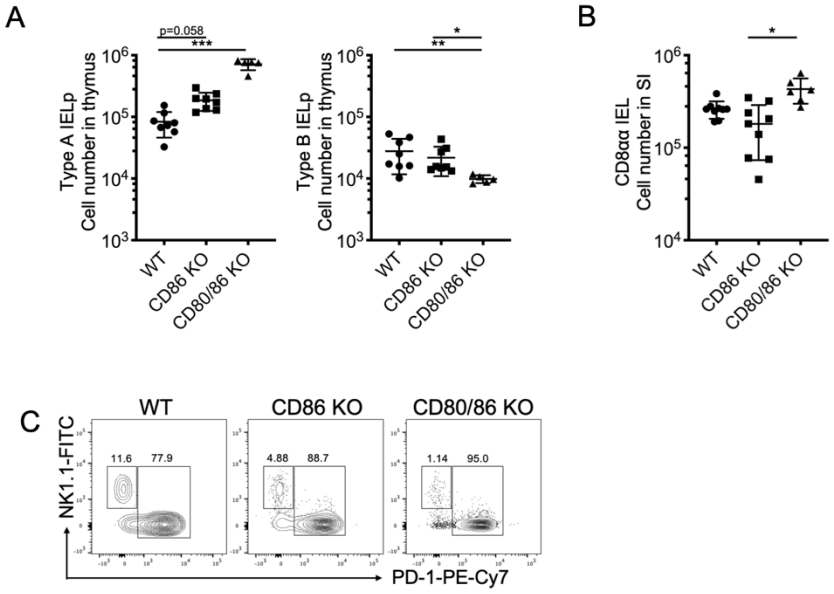

Figure 2.3. Co-stimulation by CD80 and CD86 restrains the Type A IELp fate. (A) Absolute cell number of type A IELp (left) and type B IELp (right) in WT, CD86 KO, and CD80/86 double KO mice. **(B)** Absolute number of small intestine CD8$\alpha\alpha$ IEL in WT, CD86 KO, and CD80/CD86 KO mice. **(C)** Representative data of expression of NK1.1 (Type B) and PD-1 (Type A) on CD122^{+} H2-K^{b+} mature thymic IELp in WT, CD86 KO, and CD80/86 double KO mice. Each symbol in (A and B) represents an individual mouse. Data are pooled from 4 independent experiments. Error bars show mean ± SD. *p≤0.05, **p≤0.01, ***p≤0.001, ANOVA with multiple comparisons.

2.2.3 Several subsets of hematopoietic APC express low levels of CD80

Thymocyte selection is orchestrated by both hematopoietic and stromal derived APC, but these APC subsets express varying levels of co-stimulation, with CD80 being more broadly expressed than CD86 overall[7, 9]. In light of our observation that hAPC were required for IELp selection and that CD80 co-stimulation restrained the Type A IELp fate, we hypothesized that the APC that selects Type A IELp is a hematopoietic cell that expresses MHCI but does not express CD80 or CD86. While the majority of Type A IELp reside in the thymic cortex, some can be found in the medulla[36]. Therefore, we did a comprehensive examination of the various APC found in the thymus. We included not only the well-defined dendritic cell subsets – plasmacytoid DC (pDC) and the two conventional DC (cDC) populations defined by XCR1 (cDC1) or SIRPα (cDC2) – but also B cells and various myeloid cells, as they have all been implicated in thymocyte selection or activation[9, 17, 19, 54, 55]. Thymic myeloid populations have been described separately[12, 17, 18, 56], so our APC gating strategy was designed to be comprehensive, and included several lineage markers such Siglec F (eosinophils) and Ly6G (neutrophils). As we found thymic F4/80$^+$ cells are CD64$^+$ (data not shown), CD64 was used to define thymic monocytes and macrophages after excluding eosinophils, which are F4/80$^+$ as well (gating for thymic APC shown in Fig. 2.4). These myeloid populations, such as thymic eosinophils, are not trivial in the landscape of thymic APC, and are numerically some of the most abundant (Fig. 2.5A).

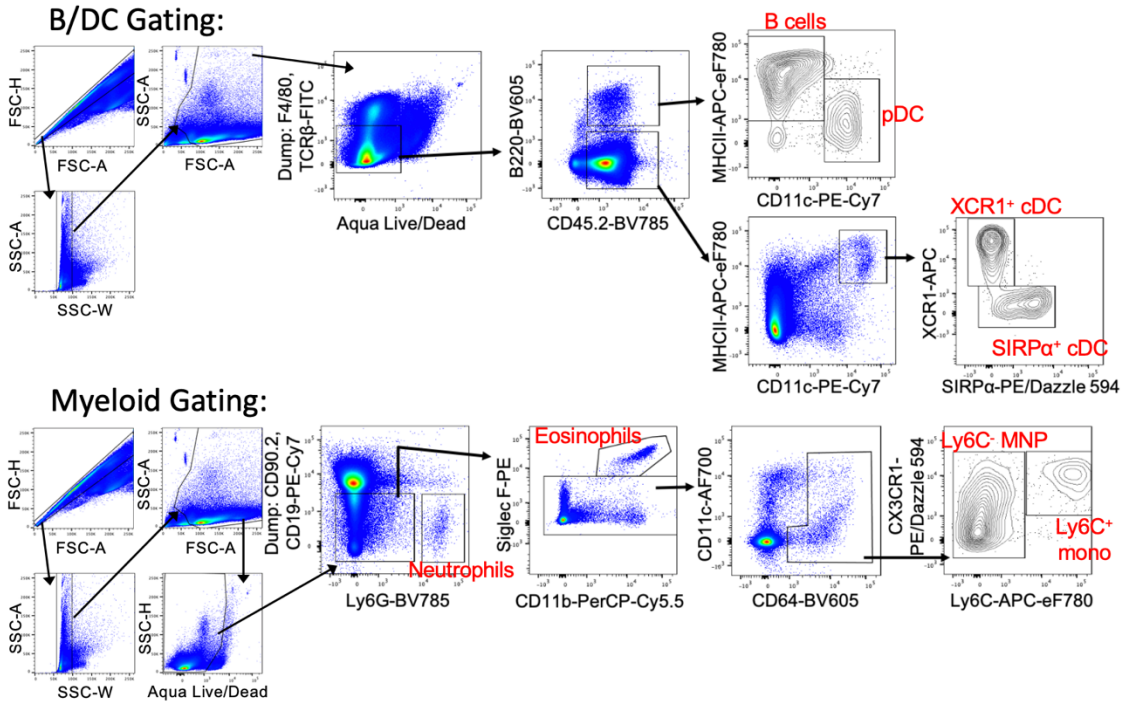

Figure 2.4. Gating for thymic APC. Representative flow cytometry gating strategy for thymic hAPC. Top panels show gating for B cells and dendritic cells: pDC, XCR1$^+$ cDC, and SIRPα^+ cDC. Bottom panels show gating for myeloid populations: neutrophils, eosinophils, Ly6C$^-$ mononuclear phagocytes (MNP, which include both macrophages and monocytes) and Ly6C$^+$ monocytes.

Four populations became appealing candidates for the APC that select Type A IELp: pDC, B cells, Ly6C$^-$ mononuclear phagocytes (abbreviated Ly6C$^-$ MNP and include thymic macrophages), and neutrophils. These four APC had low levels of CD80 and CD86 expression while expressing moderate to high levels of MHCI (Fig. 2.5B, 2.5C). Of these populations, Ly6C$^-$ MNP were of particular interest, as they were one of the most abundant APC found in the thymus (Fig. 2.5A), are of hematopoietic origin, and are localized both within the medulla and the cortex[12, 56]. Conversely, the conventional DC populations expressed high levels of CD80 and MHCI, suggesting that they may oppose self-reactive thymocytes becoming Type A IELp through deletion (Fig. 2.5B, 2.5C). Thus, to distinguish the role of the various APC subsets in Type A IELp selection, we employed different Cre-recombinase transgenic strains bred to $\beta 2m^{fl/fl}$ mice to specifically target MHCI deficiency to specific thymic APC (Table 2.1).

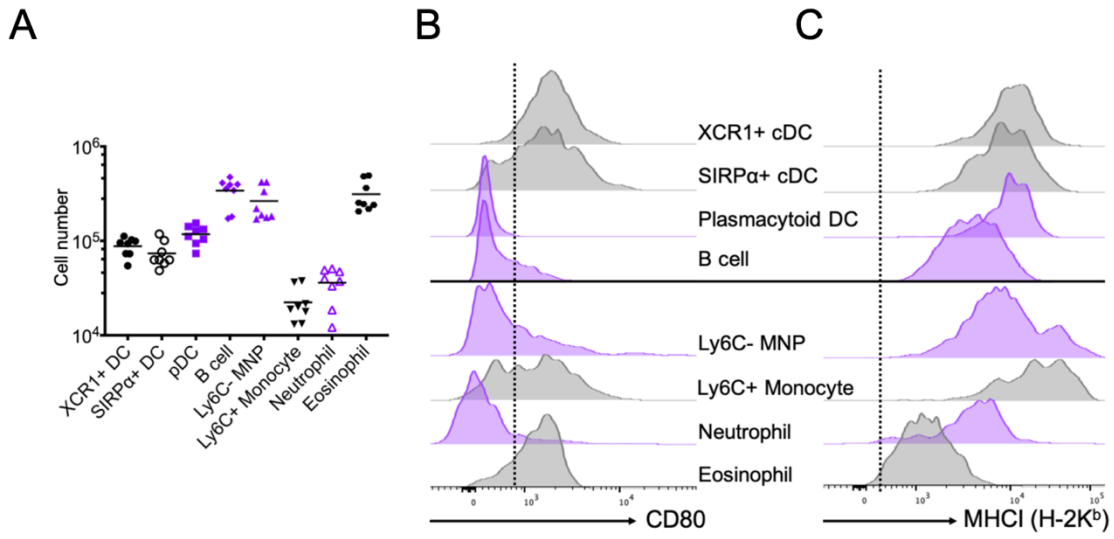

Figure 2.5. Thymic APC have heterogeneous levels of MHC class I and CD80. (A) Absolute cell number of thymic APC. Each symbol represents an individual mouse; horizontal lines indicate the mean. Data are pooled from 3 independent experiments. Representative expression of **(B)** CD80 and **(C)** H-2K^b, a classical MHCI molecule on the indicated thymic APC populations. Note that all CD86 expressing APC in the thymus also express CD80 ([7] and data not shown), thus CD80 negative APC also lack CD86. Graphs in purple indicate APC populations that are MHCI$^+$ and CD80$^-$ (pDC) or CD80^{low} (B cell, Ly6C$^-$ MNP, Ly6C$^+$ MNP).

	Zbtb46^{Cre}	*Itgax*^{Cre}	*Lyz2*^{Cre}	*Cd79a*^{Cre}
XCR1^{+} DC	+	+	-	-
SIRPα^{+} DC	+	+	-	-
pDC	-	+	-	-
B cell	-	-	-	+
Ly6C^{-} MNP	-	+	+	-
Ly6C^{+} Mono	-	-	+	-
Neutrophil	-	#	+	-
Eosinophil	-	#	-	-

Table 2.1: Conditional deletion strategy. Where "-" denotes no effect, "+" denotes significant effect, and "#" denotes mild effect in thymus

2.2.4 Conventional DCs drive cells away from the Type A IELp fate

To determine the contribution of cDC to the Type A IELp fate, we crossed $\beta 2m^{fl/fl}$ mice to zDC (*Zbtb46*) Cre to create mice that specifically targeted MHCI on SIRPα^+ and XCR1$^+$ DC (Figure 2.6A). In these zDC Cre-$\beta 2m^{fl/fl}$ mice, we observed a higher proportion and number of Type A IELp (Figure 2.7), indicating that normally, thymocyte interactions with cDC skew cells away from the IELp fate. These data fall readily in line with previous work showing that thymic conventional DC contribute to negative selection of self-reactive thymocytes and have high levels of co-stimuation[9, 54, 57].

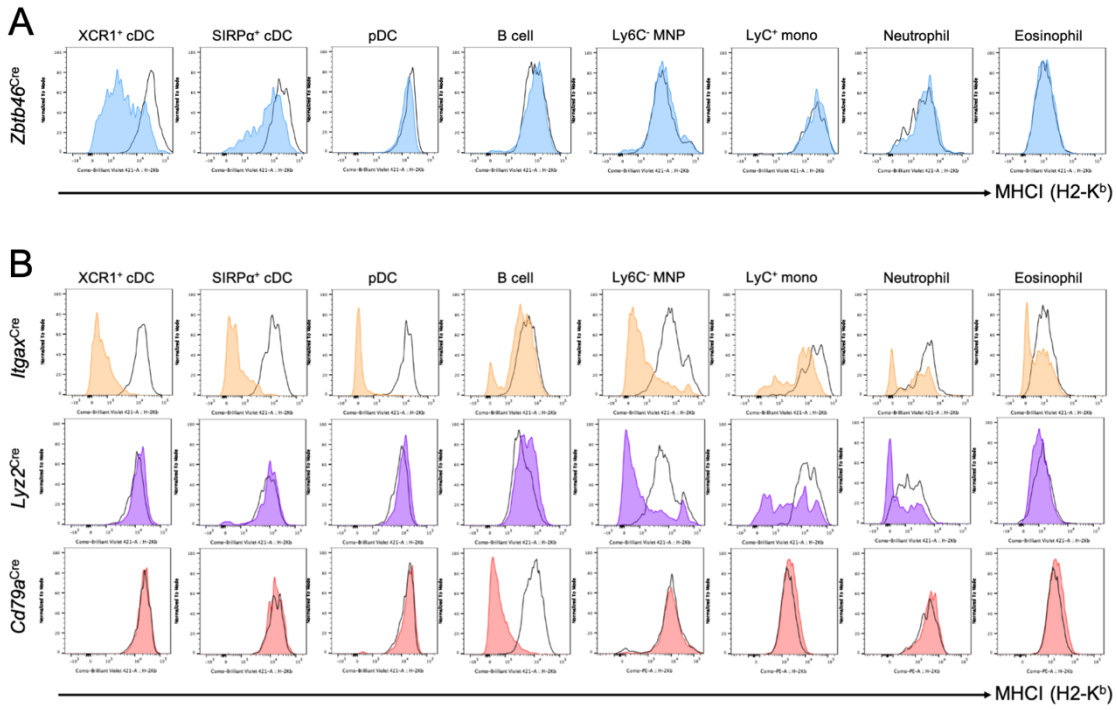

Figure 2.6. Tissue specific deletion of MHC class I using *B2m*$^{fl/fl}$. (A) Representative expression of H2-K^{b}, a classical MHCI molecule, on thymic APC in *Zbtb46*^{Cre} (zDC^{Cre})-*B2m*$^{fl/fl}$ mice (blue) and *B2m*$^{fl/fl}$ littermate controls (black). **(B)** Representative expression of H2-K^{b} on thymic APC in *Itgax*^{Cre} (CD11c^{Cre})-*B2m*$^{fl/fl}$ mice (orange), *Lyz2*^{Cre}-*B2m*$^{fl/fl}$ mice (purple), *Cd79a*^{Cre} (Mb1^{Cre})-*B2m*$^{fl/fl}$ mice (red), and *B2m*$^{fl/fl}$ littermate controls (black).

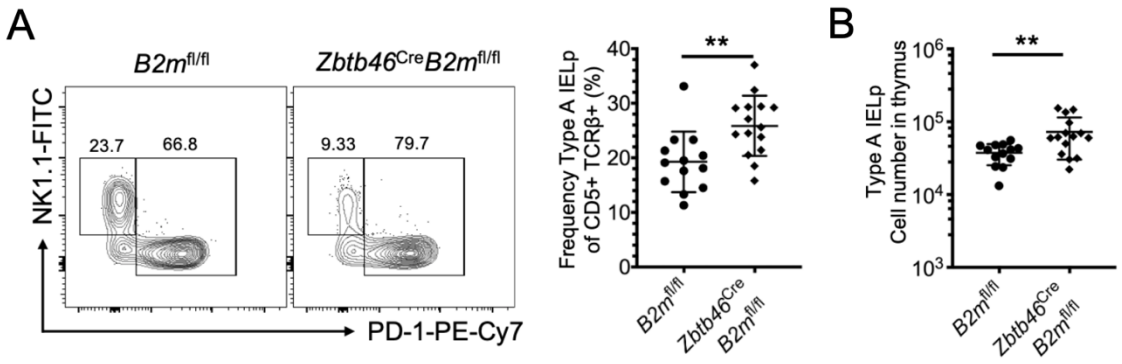

Figure 2.7. Conditional deletion of β2m in conventional DC drive more cells toward the Type A IELp fate. (A) Expression of NK1.1 (Type B IELp) and PD-1 (Type A IELp) on CD122^{+} H2-K^{b+} mature thymic IELp in *Zbtb46*^{Cre} (zDC^{Cre})-*B2m*$^{fl/fl}$ and littermate control mice (left, representative data are shown). Numbers adjacent to the outlined areas indicate the percentage of cells in each. Quantified percentage of PD-1^{+} Type A IELp in zDC^{Cre}-*B2m*$^{fl/fl}$ and *B2m*$^{fl/fl}$ mice among signaled CD5^{+} TCRβ^{+} DN thymocytes (right). **(B)** Absolute number of Type A IELp in zDC^{Cre}-*B2m*$^{fl/fl}$ and *B2m*$^{fl/fl}$ mice. Each symbol in (A & B) represents an individual mouse. Data are pooled from 7 independent experiments. Error bars show mean ± SD. **p≤0.01, unpaired two-tailed *t* test.

2.2.5 Hematopoietic APC have overlapping roles in selecting Type A IELp

Although CD11c is a well-known marker for dendritic cells, CD11c (*Itgax*) Cre targets not only cDC, but also pDC and monocytes/macrophages[58]. Using CD11c Cre-β2m$^{fl/fl}$ mice confirmed that this targeting held true for thymic APC, with lack of MHCI on cDC, pDC, and MNP; and a partial reduction on thymic eosinophils and neutrophils (Fig. 2.6B). Intriguingly, while cDC in the thymi of CD11c Cre-β2m$^{fl/fl}$ mice had robust MHCI loss, we did not see a replication of increased numbers of Type A IELp like in zDC Cre-β2m$^{fl/fl}$ thymi (Fig. 2.8A). Therefore, we reasoned that at least one of the other APC affected in the CD11c Cre-β2m$^{fl/fl}$ thymi might support Type A IELp selection. The absence of MHCI on this population could offset the increase in IELp numbers caused by fewer interactions with clonally deleting cDC, making interpretation of this result difficult.

Thus, to distinguish the role of the myeloid APC subsets, we generated LysM (*Lyz2*) Cre-β2m$^{fl/fl}$ mice. In these mice, MHCI is targeted on thymic neutrophils, Ly6C^{+} inflammatory monocytes, and Ly6C^{-} MNP, but not cDC (Fig. 2.6B). Both thymic neutrophils and Ly6C^{-} MNP expressed low levels of CD80 on their cell surface, making them attractive candidates for the APC dedicated to Type A IELp selection (Fig. 2.5B). Nonetheless, the number of Type A IELp was not affected in LysM Cre-β2m$^{fl/fl}$ mice (Fig. 2.8B). As CD11c Cre perturbed MHCI expression on thymic pDC and eosinophils as well, we depleted these APC subsets

specifically. To assess the role of pDC on selection, pDC were ablated in BDCA2 (*Clec4c)* DTR mice with diphtheria toxin (DT) administration every other day. After 9 days of DT treatment, there was no effect on Type A IELp numbers compared to BDCA2-DTR$^{-/-}$ littermate controls (Fig. 2.8C). Eosinophils were depleted using an αIL-5 antibody (clone TRFK5). After a 7-day course of αIL-5 treatment, there was no effect on Type A IELp numbers compared to controls (Fig. 2.8D). Likewise, loss of MHCI on B cells in Mb1 (*Cd79a*) Cre-β2m$^{fl/fl}$ thymi (Fig. 2.6B), did not lead to Type A IELp reduction (Fig. 2.8E).

Finally, we crossed the CD11c Cre-β2m$^{fl/fl}$ mice to Mb1 Cre to generate CD11c x Mb1 Cre-β2m$^{fl/fl}$ mice. These mice have MHCI depletion on a majority of hAPC. Thymi from these mice were not able to support normal levels of Type A IELp selection, as indicated by reduced numbers of Type A IELp compared to littermate controls (Fig. 2.8F). As single Cre models did not affect Type A IELp selection, while the dual Cre model (CD11c Cre x Mb1 Cre-β2m$^{fl/fl)}$ did, our data suggests functional redundancy amongst CD80/86 low APC for Type A IELp selection.

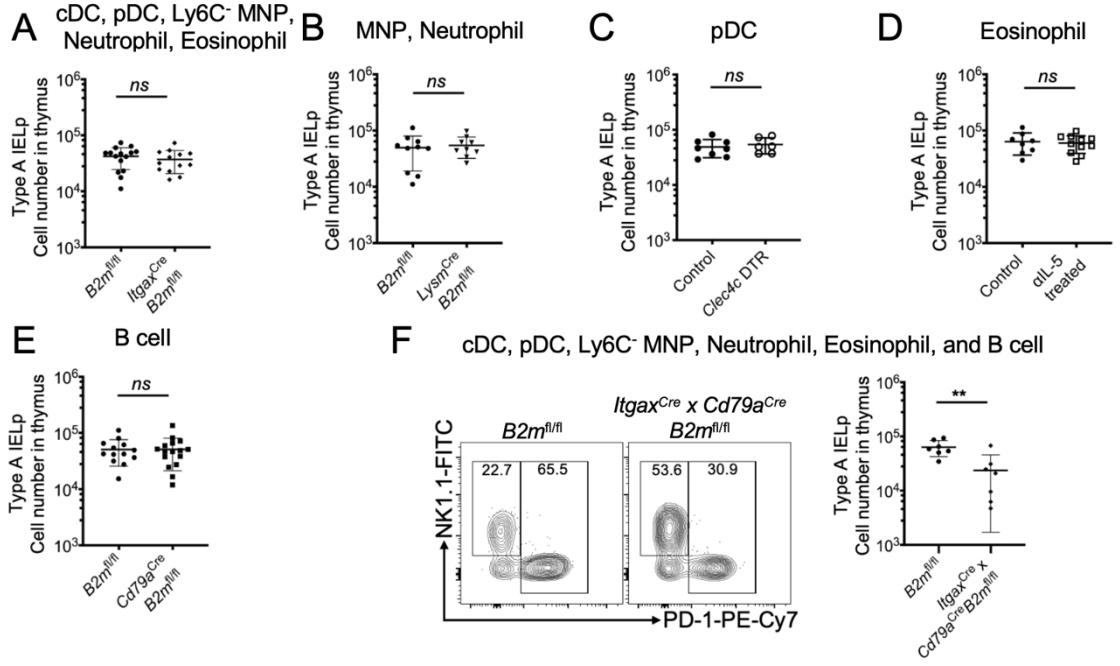

Figure 2.8. No single APC subset is dedicated to Type A IELp positive selection. (A) Absolute number of type A IELp in *Itgax*^{Cre} (CD11c^{Cre})-*B2m*$^{fl/fl}$ and littermate controls. Data are pooled from 4 independent experiments. **(B)** Absolute number of type A IELp in *Lyz2*^{Cre} (LysM^{Cre})-*B2m*$^{fl/fl}$ mice and littermate controls. Data are pooled from 3 independent experiments. **(C)** Absolute number of type A IELp in *Clec4c* (BDCA2)-DTR$^{+/-}$ mice and littermate controls after a 9d course of DT treatment. Data are pooled from 4 independent experiments. **(D)** Absolute number of type A IELp in WT mice treated with αIL-5 (clone TRFK5) or an IgG1 isotype control for 7d. Data are pooled from 3 independent experiments. **(E)** Absolute number of type A IELp in *Cd79a*^{Cre} (Mb1^{Cre})-*B2m*$^{fl/fl}$ and littermate controls. Data are pooled from 5 independent experiments **(F)** Representative data of expression of NK1.1 (Type B) and PD-1 (Type A) on CD122^{+} H2-K^{b+} mature thymic IELp (left). Absolute number of type A IELp *Itgax*^{Cre}x*Cd79a*^{Cre}-*B2m*$^{fl/fl}$ and littermate controls (right). Data are pooled from 3 independent experiments. For (A – F), the graph titles indicate the thymic APC populations affected in the experimental mice (Cre^{+}, BDCA2 DTR^{+}, αIL-5 treated). Each symbol represents an individual mouse. Error bars show mean ± SD. **p≤0.01, unpaired two-tailed *t* test.

2.3 Discussion

Type A IELp, the thymic precursors of CD8αα IEL in the gut, receive strong stimulation by self-antigens, yet escape clonal deletion[36, 39, 40, 41]. In this study, we investigated the thymic antigen presenting cells that orchestrate the decision of clonal diversion into IELp instead of deletion. Using β2m deficient bone marrow chimeras, we showed that MHCI expression on hAPC rather than stromal APC was crucial for selection of IELp.

The current and previous studies have shown that CD28-mediated co-stimulation, along with TCR stimulation, is important in determining thymocyte fate into the deletion pathway[36, 47, 48]. CD28 on developing thymocytes can interact with the ligands CD80 (B7-1) and CD86 (B7-2), but these two molecules are distinct – sharing only approximately 25% sequence identity, and are differentially expressed on various APC[7, 59]. We therefore hypothesized that the two B7 molecules serve non-redundant roles in selection. This was supported by our data in CD80/CD86 double knockout mice compared to the CD86 knockout mice. Out data support previous findings that CD80 serves a non-redundant role in mediating clonal deletion in the thymic cortex – the region in which most Type A IELp reside[7, 36]. These distinct roles may be due to differences in which APC express CD80 or CD86. Interestingly, hAPC in the thymus do not generally express CD86 alone, but rather express either CD80 alone or both CD80 and CD86. Alternatively, these distinct roles may be due to differences in the interactions between these ligands and their mutual receptor, as CD86 is

predicted to bind more strongly to CD28[60]. Without the CD80/CD86 ligand interactions, CD28 accumulates only half as well at the immunological synapse and only needs CD86 expression for the CD28 accumulation, not CD80[61].

Given that our data showed that hAPC are required for Type A IELp selection and that CD28 co-stimulation restrains this fate, we examined the role of thymic APC that expressed MHCI with little/no CD80, or high levels of CD80. Similar to previous reports, we saw that thymic conventional DC expressed high levels of co-stimulatory molecules, which contributes to their role in deletional tolerance[9, 11]. When we selectively depleted MHCI on cDC, so that class I restricted, self-reactive developing thymocytes could no longer interact with these 'deleting' APC, we indeed found that more cells were diverted toward the Type A IELp fate. On the other hand, thymic pDC, Ly6C$^-$ MNP, B cells, and neutrophils expressed high levels of MHCI but minimal levels of CD80, suggesting a role in IELp positive selection. However, when we selectively depleted MHCI on each population individually, we did not see a decrease in Type A IELp selection as expected if that population was essential. Instead, we only found a decrease in Type A IELp when MHCI was depleted on the majority of hAPC, suggesting functional redundancy amongst CD80/86 $^{neg/low}$ APC in contributing to the diversion fate.

Like thymic hAPC, DP thymocytes do not express CD80 or CD86, and it could be reasoned that they could potentially select IELp as well. Indeed, iNKT cells,

another relatively self-reactive population, are selected on cortical DP thymocytes expressing lipid antigens on CD1d [33]. However, DP thymocytes express very low levels of classical MHCI on their cell surface. Further, our results showed a decrease in IELp selection when classical MHCI was depleted on thymic APC alone. Thus, DP thymocytes, which represent the largest population of cells in the thymus, are an unlikely candidate for IELp selection. While CD1d itself is β2m dependent and was also likely depleted with classical MHCI in our cell-specific deletion of β2m, Type A IELp are not dependent on CD1d for selection[36, 62].

In conclusion, we showed that hAPC play an essential role in clonal diversion into the IELp fate, although it appears there is no single APC that is specialized for 'diverting' thymocytes to Type A IELp. In addition, the loss of co-stimulatory molecules on all APC exaggerates Type A IELp selection, while the presence of cDC with high levels of co-stimulatory molecules restrains it.

2.4 Materials and Methods

2.4.1 Mice

C57BL/6NCrl (B6) and B6.SJL-Ptprc^{a}Pepc^{b}/BoyCrl (B6.SJL) mice were purchased from the National Cancer Institute. B6.129P2-*B2m*^{tm1Unc}/DcrJ (*B2m*$^{-/-}$), B6.129S4-*Cd80*^{tm1Shr} *Cd86*^{tm2Shr}/J (CD80/CD86 KO), B6.Cg-*Zbtb46*$^{tm3.1(cre)Mnz}$/J (*Zbtb46*^{Cre}), B6.Cg-Tg(Itgax-cre)1-1Reiz/J (*Itgax*^{Cre}), B6.C(Cg)-*Cd79a*$^{tm1(cre)Reth}$/EhobJ (*Cd79a*^{Cre}), and C57BL/6-Tg(CLEC4C-HBEGF)956Cln/J (*Clec4c* DTR) mice were obtained from Jackson Laboratories. *Cd80*^{flox}*BACTg*/ B6.129S4-*Cd80*^{tm1Shr} *Cd86*^{tm2Shr}/J, referred to as CD86 KO mice in this study, were kindly provided by R. J. Hodes (National Institutes of Health) and were described previously[63]. B6.*B2m*$^{flox/flox}$ (*B2m*$^{fl/fl}$) mice were kindly provided by C. N. Morrell (University of Rochester School of Medicine) and were described previously[64]. B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J (Lyz2^{Cre}) mice were kindly provided by M. Jenkins (University of Minnesota). Except the bone marrow chimeras, mice were used between 6 to 10 weeks of age, and were age-matched with controls in each experiment. Animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

2.4.2 Bone marrow chimeras

For bone marrow chimeras, recipients were depleted of NK cells with PK136, then lethally irradiated and reconstituted with donor bone marrow depleted of T cells. Chimeras were provided water supplemented with neomycin and polymyxin

B for 2 weeks. Chimeras involving *B2m*$^{-/-}$ recipients and bone marrow were analyzed at a minimum of 8 weeks after reconstitution.

2.4.3 Lymphocyte isolation

Single cell suspensions of thymocytes were isolated by mashing and filtering thymi through 70μM cell strainers (Falcon), or digestion with collagenase D (Roche, 1mg/mL) at 37°C for 30 minutes before mashing and filtering thymi through 70μM cell strainers. Small intestine IEL were isolated as previously described[36] and filtered through 70μM cell strainers. Lymphocytes were enriched by centrifugation over a 80%:40% Percoll gradient.

2.4.4 Flow cytometry

Single cell suspensions were incubated with Fc block (Tonbo) for 15 minutes at 4°C before staining with surface antibodies and viability dye for 30 minutes at 37°C or 1 hour at room temperature. Antibodies from BioLegend were: CD4 (RM4-5), CD8b (YTS156.7.7), CD25 (PC61), TCRγδ (GL3), NK1.1 (PK136), CD45.2 (104), B220 (RA3-6B2), CD11c (N418), XCR1 (ZET), SIRPα (P84), CD90.2 (30-H12), CD19 (6D5), Ly6G (1A8), CD64 (X54-5/7.1), CX3CR1 (SA011F11), and CD80 (16-10A1). Antibodies from BD Biosciences were: CD8α (53-6.7), TCRβ (H57-597), H2-K^{b} (AF6-88.5), Siglec F (E50-2440), and CD86 (GL1). Antibodies from eBioscience were: CD5 (53-7.3), PD-1 (J43), CD122 (TM-b1), F4/80 (BM8), MHC Class II (I-A/I-E, clone M5/114.15.2), and Ly6C (HK1.4). CD11b (M1/70) was purchased from Tonbo. Biotinylated CD1d–PBS57

monomers were obtained from the US National Institutes of Health tetramer core and were incubated with APC streptavidin to tetramerize. Samples were acquired on a BD Fortessa or BD LSRII, and data were analyzed with FlowJo 10.

2.4.5 APC subset depletion treatments

For pDC depletion, *Clec4c* DTR and littermate controls were injected intraperitoneally (i.p.) with diphtheria toxin (DT; Sigma-Aldrich) in 100uL PBS every other day for 9 days (500ng DT for first injection, 100ng of DT for subsequent injections). For eosinophil depletion, B6 mice were treated i.p. every other day for 7 days with 25μg αIL-5 (TRFK5) or IgG1 isotype control (HRPN) in 100uL PBS. Tissues for treated mice were harvested the day after the final injections.

2.4.6 Statistical analysis

Data were analyzed using GraphPad Prism software version 8.0. For comparison of two data sets, two-tailed, unpaired *t* tests were performed. For comparisons of three or more data sets, 1-way ANOVA with multiple comparisons was used for data analysis and calculation of *p* values. *P* values ≤ 0.05 were considered significant. Numbers of experimental replicates and additional details are explained in each figure legend.

2.5 Contributions

STL, RR, and KAH designed the experiments. STL, HG, ERB, and RR performed the experiments. STL and RR analyzed the data. STL and KAH wrote the manuscript with input from all the authors.

Chapter 3

Early life thymic IFNβ is driven by AIRE and changes thymic APC composition

3.1 Introduction

Type I interferons (IFN-I), which include IFNα and IFNβ, are cytokines that are rapidly produced during viral infections and contribute to an effective anti-microbial response[65]. IFN-I exposure leads to a wide breadth of immunological effects, mediated by various interferon stimulated genes (ISG). Not surprisingly, IFN-I is tightly regulated[66]. Type I interferon dysregulation has been associated with a number of autoimmune disorders, including systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis, indicating a relationship between interferons and self-tolerance[67, 68]. Interferon production and signaling is not only found during infection, but also at low levels at the steady state[69].

Tonic levels of IFNβ are particularly high in the thymus, where developing T cells undergo selection processes that remove self-reactive clones from the repertoire[29]. Correspondingly, developing T cells continually respond to thymic interferon[30]. In the absence of this signal, mature CD4 and CD8 single positive (SP) thymocytes fail to upregulate Qa2, a marker often used to define more mature SP thymocytes[30, 70]. Clonal deletion of self-reactive SP clones was initially thought to mostly occur at an earlier semi-mature stage, but more recent work in using a polyclonal repertoire indicates the deletion occurs in mature SP thymocytes as well[7, 71].

SP thymocytes are generally found in the inner, medullary area of the thymus, where negative selection of auto-reactive cells is mediated by antigen presenting cells like medullary thymic epithelial cells (mTEC) and dendritic cells[5]. A subset of mTEC express autoimmune regulator (AIRE), driving expression of tissue-restricted antigens (TRA) – a step critical for self-tolerance. Intriguingly, tonic IFNβ is produced by mTEC in the thymus, rather than by plasmacytoid dendritic cells or other antigen presenting cells (APC) that often produce IFN-I in response to viral pathogens[29, 72, 73]. The relationship between AIRE$^+$ mTEC and IFNβ remains controversial, however, making the role of thymic interferon on T cell tolerance difficult to define[29, 72]. Given this, we sought to better describe the expression of thymic IFNβ and its effect on thymic antigen presenting cells. Using both IFNβ reporter and knockout mice, we show that thymic IFNβ is produced by a small subset of mTEC, and this expression is AIRE-dependent. We also report that both AIRE and IFNβ have an age-dependent component to their expression, independent of overall thymic size – peaking early in life. Further, both thymocytes and thymic APC respond to IFNβ, resulting in changes in thymic composition in the absence of tonic interferon signaling. Collectively, these findings provide valuable insight into the role of IFN-I on thymic APC, shedding light on its potential effects on T cell tolerance.

3.2 Results

3.2.1 Thymic interferon-β is produced by a subset of mTEC^{hi} cells at the steady state

While IFNα consist of several isoforms encoded by 14 distinct genes, IFNβ is encoded by a single gene[66]. To determine if developing thymocytes are capable to responding to IFNβ specifically, we made use of both IFNβ-deficient (*Ifnb1*$^{-/-}$) and IFN-I receptor deficient (*Ifnar1*$^{-/-}$) mice. Like previous reports that indicated that Qa2 upregulation was dependent on type I interferon signaling, we found lower levels of Qa2 in CD4SP thymocytes in both IFNβ and receptor deficient mice, even in the absence of infection (Figure 3.1a, left panels). The decrease of Qa2 expression was similar between the *Ifnb*$^{-/-}$ and *Ifnar1*$^{-/-}$ strains, suggesting that IFNβ alone makes a large contribution to the type I interferon signaling in thymocytes. Besides type I interferons, there are other interferon families: type II and type III, all three of which signal through STAT1[74]. STAT1 deficient (*Stat1*$^{-/-}$) mice showed the deepest reduction in Qa2 levels, indicating interferon signaling through other pathways as well (Figure 3.1a, left panels). Like Qa2, Ly6A (Sca-1) is used to define mature thymocytes[75], and Ly6A expression was also decreased in the absence of interferon signaling (Figure 3.1a, right panels). Like their CD4SP counterparts, CD8SP thymocytes showed interferon conditioning, with lower levels of both markers in the *Ifnb1*$^{-/-}$, *Ifnar1*$^{-/-}$, and *Stat1*$^{-/-}$ mice (Figure 3.1b).

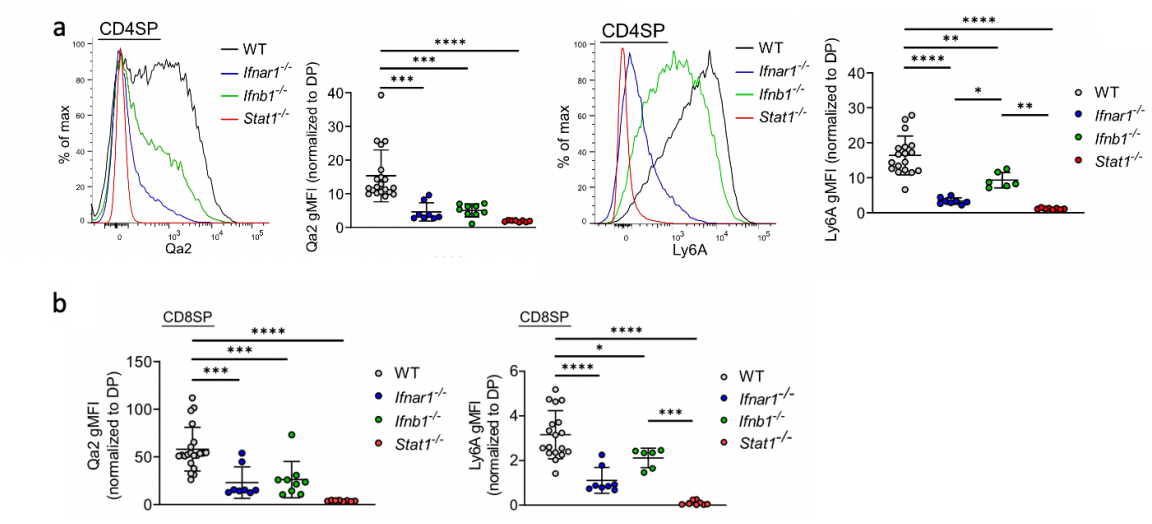

Figure 3.1. Developing T cells respond to IFNβ. (a) Representative expression of Qa2 on CD4SP thymocytes from WT, *Ifnar1*$^{-/-}$, *Ifnb1*$^{-/-}$, and *Stat1*$^{-/-}$ mice (left). Qa2 geometric mean fluorescence intensity (gMFI) of CD4SP normalized to mean DP Qa2 gMFI (2nd panel). Representative expression of Ly6A on CD4SP thymocytes from WT, *Ifnar1*$^{-/-}$, *Ifnb*$^{-/-}$, and *Stat1*$^{-/-}$ mice (3rd panel). Ly6A gMFI of CD4SP normalized to mean DP Ly6A gMFI (4th panel). **(b)** Qa2 (left) and Ly6A (right) gMFI of CD8SP normalized to corresponding DP gMFI. Each symbol in (a & b) represents an individual mouse. Error bars show mean ± SD. *p≤0.05, **p≤0.01, ***p≤0.001, ****p ≤ 0.0001, ANOVA with multiple comparisons.

Previous reports have suggested that thymic epithelial cells, particularly mTEC, produce IFNβ[29, 72]. Using IFNβ reporter mice in which IFNβ has been replaced by firefly luciferase (Δβ-luc-mice[29]), we confirmed that $CD45^-$ thymic epithelial cells express higher levels of IFNβ compared to their $CD45^+$ counterparts (Figure 3.2a). The highest levels of IFNβ was seen in $CD45^-$ $CD80^+$ cells, a population that encompasses the $mTEC^{hi}$ subset – $mTEC^{hi}$ express high levels of both MHCII and CD80, while cortical thymic epithelial cells (cTEC) and $mTEC^{lo}$ do not express CD80[76] (Figure 3.2a). This was also reflected in the highest levels of IFNβ transcript in $mTEC^{hi}$ sorted from wild-type mice (Figure 3.2b). Using a second reporter in which an IRES-tdTomato was knocked into the IFNβ locus ($IFN\beta^{KI/KI}$), we further found that only a very small subset of $mTEC^{hi}$ are producing IFNβ (Figure 3.2c). Taken together, our data suggest that the type I interferon signaling seen in developing thymocytes can be attributed to low amounts of tonic IFNβ produced by a subset of $mTEC^{hi}$.

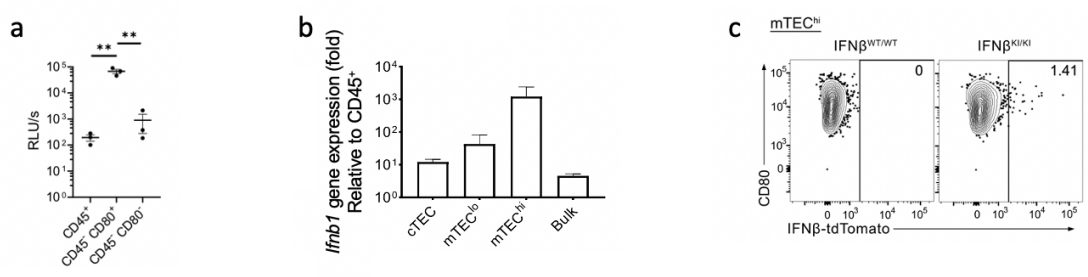

Figure 3.2. IFNβ is produced by mTEC^{hi}. (a) Luminescence of indicated cell populations (enriched using MACS bead enrichment) from thymi of Δβ-luc-mice. Each symbol represents an individual mouse. Data are pooled from 2 independent experiments. Error bars show mean ± SD. **p≤0.0, ANOVA with multiple comparisons. **(b)** *Ifnb1* expression by qPCR in sorted cTEC, mTEC^{lo}, mTEC^{hi}, and total Liberase TH digested thymus (bulk) from wild-type B57BL/6 mice (n=4) presented relative to that of sorted CD45^{+} cells from the same experiment. Error bars show mean ± SD. **(c)** Representative data of expression of IFNβ-tdTomato in mTEC^{hi} from control (left) or reporter (right) strains.

3.2.2 Interferon-β is dependent on continuous AIRE expression

While the majority of mTEC will express AIRE at some point during their differentiation, AIRE expression is restricted to a fraction of mTEC^{hi} at any given time[77]. Of AIRE^{+} mTEC, only a subset will express any particular tissue-restricted antigen, leading to expression of these antigens by very few mTEC – similar to the expression pattern of thymic IFNβ[5]. Like with TRA, we found that IFNβ expression was AIRE dependent, resulting in lower *Ifnb* transcripts in sorted mTEC^{hi} samples from AIRE deficient mice compared to WT controls (Figure 3.3a). These data are in line with previous work using the Δβ-luc-mice on an AIRE deficient background[29]. Corresponding to the absence of IFNβ produced by mTEC, developing CD4SP and CD8SP show a phenotypic lack of interferon conditioning in AIRE deficient mice (Figures 3.3b and 3.3c).

For development of AIRE^{+} mTEC, RANK-RANKL signaling is critical[78]. Therefore, we treated mice with a single dose of blocking anti-RANKL antibody to determine if diminished AIRE expression would lead to reduced levels of IFNβ. Only 48 hours after the Δβ-luc-mice were treated, we saw significantly reduced expression of IFNβ (Figure 3.3d). As expected, the RANKL blockade reduced AIRE^{+} mTEC^{hi}, without depleting AIRE^{-} mTEC^{hi}, mTEC^{lo}, or cTEC (Figure 3.3e and data not shown). The decrease of AIRE^{+} mTEC in the RANKL treated mice could be due to the reduction of IFNβ rather than the converse. However, when we looked at *Ifnb1*$^{-/-}$ and *Ifnar*$^{-/-}$ mice, we did not see a significant reduction in any thymic epithelial cell subset, indicating that differentiation is not dependent on

IFN-I signals (Figure 3.4), as previously suggested[72]. Rather, IFNβ expression by mTEC^{hi} is dependent on continuous RANKL signaling and AIRE.

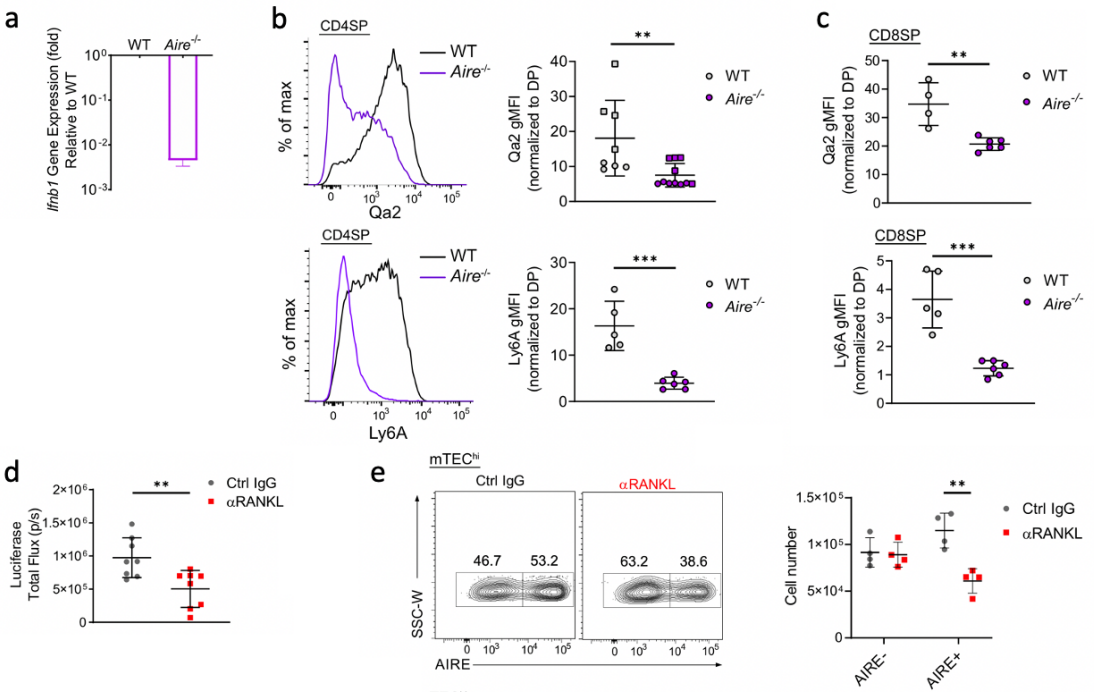

Figure 3.3. IFNβ expression is AIRE dependent. (a) *Ifnb1* expression by qPCR in sorted mTEC^{hi} from WT and *Aire*$^{-/-}$ mice (n=2) presented relative to WT mTEC^{hi}. Error bars show mean ± SD. **(b)** Representative expression of Qa2 on CD4SP thymocytes from WT and *Aire-/-* mice; and Qa2 gMFI of CD4SP normalized to mean of DP Qa2 gMFI (top row). Representative expression of Ly6A on CD4SP thymocytes from WT and *Aire*$^{-/-}$ mice; and Ly6A gMFI of CD4SP normalized to mean of DP Ly6A gMFI (bottom row). **(c)** Qa2 (top) and Ly6A (bottom) gMFI of CD8SP normalized to corresponding DP gMFI. **(d)** Bioluminescence (as measured by average flux in photons/second) from *in vivo* imaging of Δβ-luc-mice injected with D-Luciferin (48 hours after treatment with a control or αRANKL antibody). Data are pooled from 4 independent experiments. **(e)** Representative data of expression of AIRE by flow cytometry in mTEC^{hi} from control IgG or αRANKL treated mice, 48h after treatment (left). Absolute cell

number of thymic AIRE^{+} mTEC (right). Data are pooled from 2 independent experiments. Each symbol in (b-e) represents an individual mouse. Error bars show mean ± SD. **p≤0.01, ***p≤0.001, unpaired two-tailed *t* test.

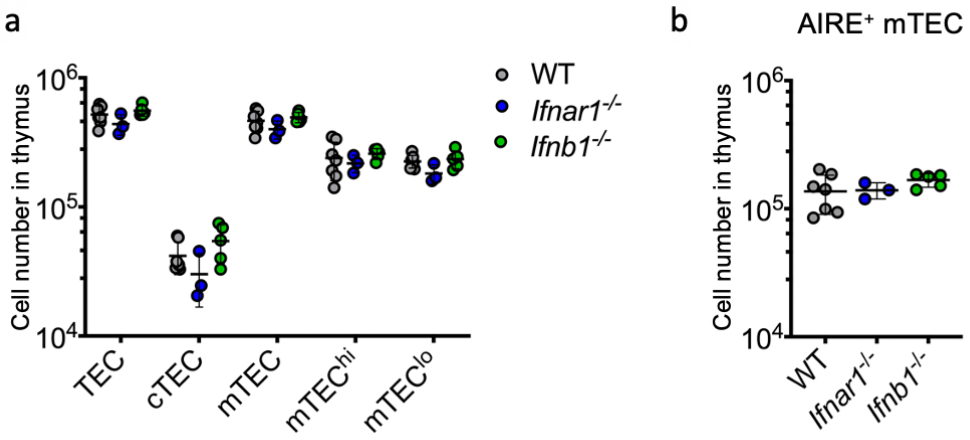

Figure 3.4. mTEC differentiation is not dependent on type I interferon. (a) Absolute number of TEC, cTEC, mTEC, mTEC^{hi}, and mTEC^{lo} in WT, *Ifnar1*$^{-/-}$, and *Ifnb1*$^{-/-}$ mice. **(b)** Absolute number of AIRE^{+} mTEC in WT, *Ifnar1*$^{-/-}$, and *Ifnb1*$^{-/-}$ mice. Data are pooled from 2 independent experiments. Each symbol in (a & b) represents an individual mouse. Error bars show mean ± SD.

3.2.3 Interferon-β production and AIRE expression peak early in life

Immune responses change with age, characterized generally by declining immune function in the elderly[79]. There is increased susceptibility to viral infections in which production of interferons provides a protective effect, and, indeed, dendritic cells like plasmacytoid DC have an impaired capacity of type I interferon production in response to infection in aged individuals[80]. We reasoned that tonic IFN-I production in the thymus could also have an age component. By tracking the IFNβ signal in the Δβ-luciferase reporter mice over time, we saw the highest levels of IFNβ at approximately 3 weeks of age before dropping and plateauing by 8 weeks (Figure 3.5a). As thymic cellularity and composition is also known to have an age component[81, 82], we tracked the number of mTEC with age. Overlapping with the IFNβ expression in the thymus, the proportion of $AIRE^+$ mTEC to all other thymic populations was highest at 3 weeks (Figure 3.5b). This was driven by the high overall numbers of $AIRE^+$ mTEC in early life, as age-related thymic involution does not occur until adulthood and thymic size is continues to increase after 3 weeks (Figure 3.6; data not shown for overall thymic cellularity). Thus, the age dependence of thymic interferon expression is likely due to the fluctuation in AIRE expression.

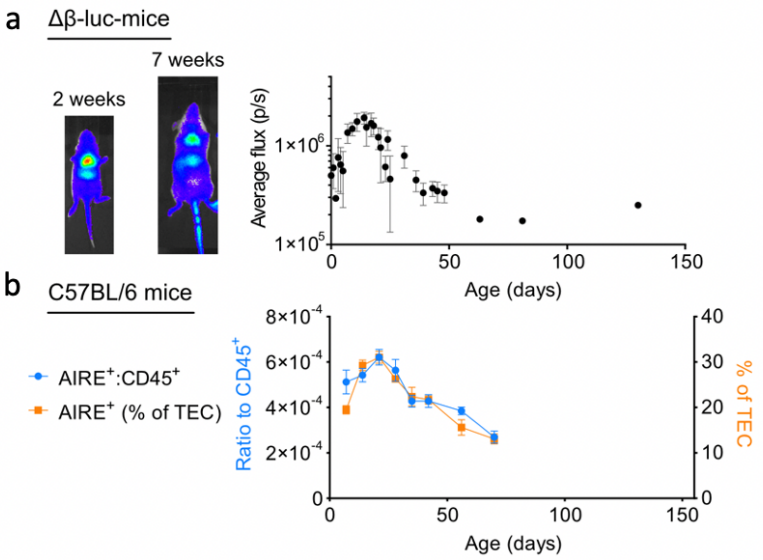

Figure 3.5. Peak IFNβ expression at 3 weeks of age corresponds to a peak in AIRE⁺ mTEC. (a) Representative *in vivo* images of Δβ-luc-mice injected with D-Luciferin at 2 weeks and 7 weeks of age (left). Bioluminescence (as measured by average flux in photons/second) from *in vivo* imaging of Δβ-luc-mice injected with D-Luciferin at different ages. Between 1 to 8 mice per time point; error bars show mean ± SD. **(b)** AIRE⁺ mTEC as a ratio to CD45⁺ cells (left axis, circles) or as a proportion of TEC (right axis, squares) in various ages of WT C57BL/6 mice. Data are pooled from 14 independent experiments, with at least 2 experiments and 6 individual mice per timepoint. Error bars show mean ± SEM.

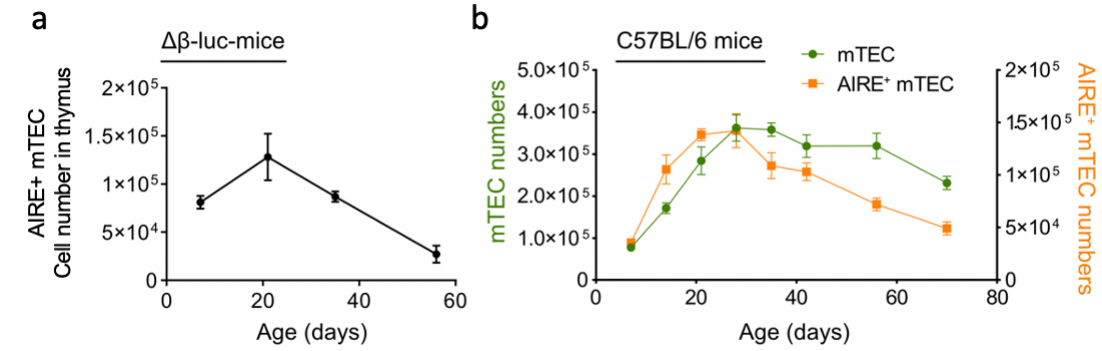

Figure 3.6. TEC numbers in Δβ-luc (IFNβ reporter) and C57BL/6 mice. (a) Absolute number of AIRE$^+$ mTEC in various ages of Δβ-luc-mice. Data are pooled from 2 independent experiments with n=2-3 per timepoint. Error bars show mean ± SEM. **(b)** Absolute number of mTEC (left axis, circles) or AIRE$^+$ mTEC (right axis, squares) in various ages of WT C57BL/6 mice. Data are pooled from 14 independent experiments, with at least 2 experiments and 6 individual mice per timepoint. Error bars show mean ± SEM.

3.2.4 Both thymocytes and thymic APC respond to type I interferons

Although there are many subtypes of Type I interferons, they all share a heterodimeric receptor composed of 2 chains: IFN-α receptor 1 (IFNaR1) and IFN-α receptor 2 (IFNaR2). This receptor is expressed ubiquitously on all nucleated cells[83]. Nonetheless, different cell types have distinct interferon responses: for example, in response to influenza, interferon responses – as measured by activation of Mx1, an interferon-stimulated gene – are contained mostly within the hematopoietic compartment and is especially high within Ly6C$^+$ monocytes[84]. In the thymus, the hematopoietic APC compartment includes dendritic cells, B cells, and various myeloid populations, many of which have been implicated in thymocyte selection or activation[9, 17, 19, 54, 55]. Using Mx1^{GFP} reporter mice, we observed tonic interferon signaling in the majority of cells in the thymus, with the exception of neutrophils and eosinophils (Figure 3.7a; gating shown Figure 2.4). When these Mx1^{GFP} reporters were bred onto to an *Ifnar1*$^{-/-}$ background, we saw reduced levels of GFP, indicating that these cells normally respond to type I interferon (Figure 3.7a and 3.7b). When crossed to a *Stat1*$^{-/-}$ background, these reductions were deeper and broader, suggesting that IFN-II or -III signaling may shape the APC compartment as well, particularly for B cells and XCR1$^+$ DC (Figure 3.7a and 3.7c). Without type I interferon signaling in the thymus, there were significant reductions in the number of hematopoietic APC, particularly in pDC and Ly6C$^+$ monocytes (Figure 3.8). Again, these changes to APC were deeper and broader when all interferon signaling was abolished in

Stat1$^{-/-}$ mice, suggesting that non-IFN-I signaling also shapes the APC compartment (Figure 3.8).

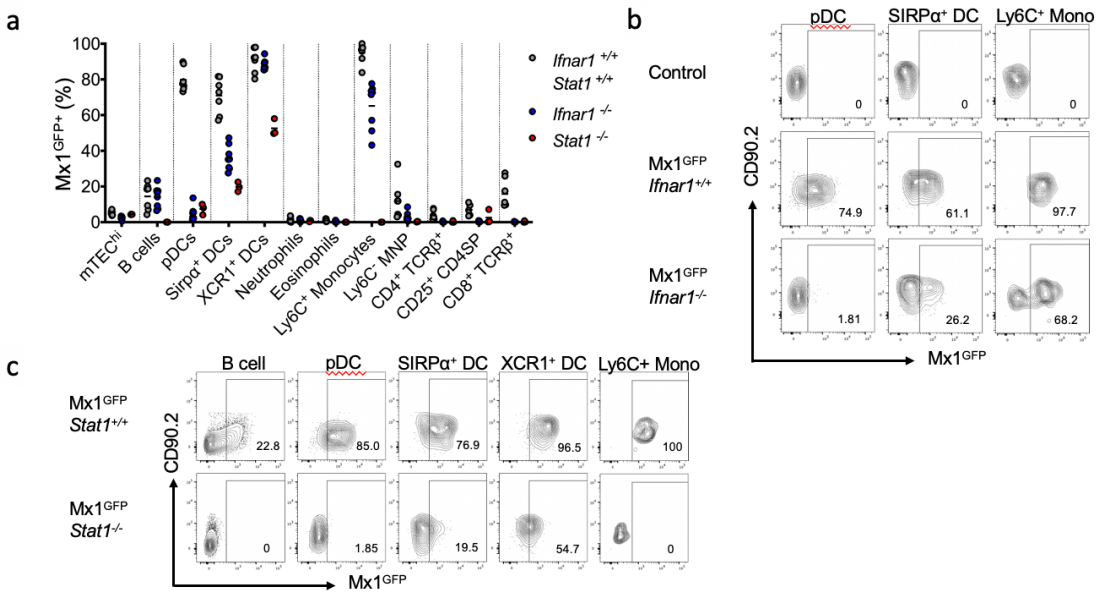

Figure 3.7. **Most cells in the thymic environment respond to type I interferons at the steady state. (a)** Quantified percentage of GFP^{+} cells of various thymic populations in Mx1^{GFP} reporter mice on wildtype, *Ifnar1*$^{-/-}$, or *Stat1*$^{-/-}$ backgrounds. **(b)** Representative data of expression of Mx1^{GFP} in various hematopoietic APC from control wildtype C57BL/6; Mx1GFP-*Ifnar1*$^{+/+}$, and Mx1GFP-*Ifnar*$^{-/-}$ mice. Only APC with the largest change between the *Ifnar1*$^{+/+}$ and *Ifnar*$^{-/-}$ strains are shown. **(c)** Representative data of expression of Mx1^{GFP} in various hematopoietic APC from Mx1GFP-*Stat*$^{+/+}$, and Mx1GFP-*Stat*$^{-/-}$ mice. Only APC with the largest change between the *Stat*$^{+/+}$ and *Stat*$^{-/-}$ strains are shown. Like in Figure 3.7b, GFP^{+} gates were determined using a non-transgenic C57BL/6 control.

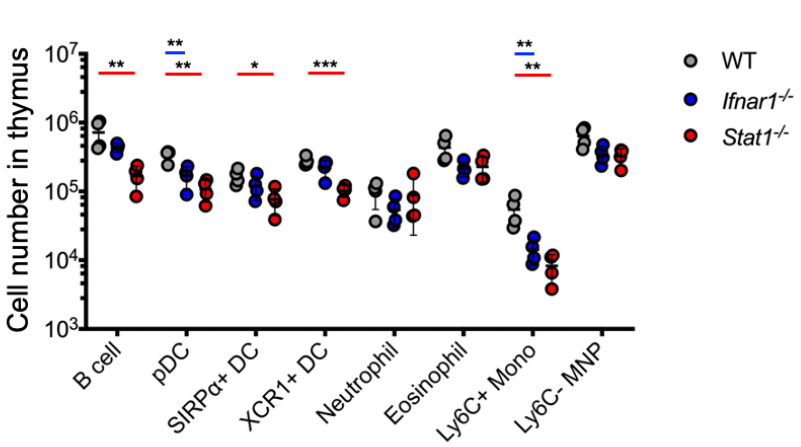

Figure 3.8. Thymic interferon changes hematopoietic APC composition. Absolute number of various hematopoietic APC in WT, *Ifnar1*$^{-/-}$, and *Stat1*$^{-/-}$ mice. Data are pooled from 2 independent experiments. Each symbol represents an individual mouse. Error bars show mean ± SD.

3.3 Discussion

Type I interferons are typically thought of in the context of viral infections, but more recently, their constitutive expression has been noted at low levels in some organs like the thymus[29, 65, 72, 84]. In this study, we examined the source of thymic IFNβ and its effect on thymic APC. Using both IFNβ reporters and subset enrichments, we showed that mTEC^{hi} produce IFNβ at the steady state in an AIRE dependent fashion. A transcriptional regulator, AIRE allows mTEC^{hi} to induce expression of various tissue restricted antigens – a role that is critical for negative selection in thymus[5]. The ability of AIRE to promote promiscuous gene expression is mediated by its various molecular partners, many of which are still elusive[85]. Similar to the expression of TRA by AIRE^{+} mTEC^{hi}, we found that only a small subset of mTEC^{hi} expressed IFNβ, rather than continuous expression by all cells.

The importance of AIRE expression is seen in mice and humans that have *Aire* mutations: both have autoantibody production and multi-organ autoimmune manifestations[86, 87, 88]. In humans, mutations in *Aire* lead to autoimmune diseases, particularly in endocrine organs, and is referred to as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) or autoimmune polyendocrine syndrome 1 (APS-1). Intriguingly, antibodies against type I interferons, both IFNα and IFNβ, can be found in APS-1 patients[89].

During viral infections, the response by APC to IFN-I includes the upregulation of MHC and costimulatory molecules on their cell surface, which promotes robust T cell responses[90]. Similarly, type I interferon signaling in thymic APC may promote antigen presentation by driving expression of related genes, thereby mediating thymic selection processes. As well as thymic IFN-I, there is also tonic type III interferon (IFNλ) expression by mTEC[91]. This thymic IFNλ enhances MHC class I molecules on both cTEC and mTEC, and *Ifnlr1*$^{-/-}$ mice are characterized by both aberrant thymic selection and autoantibody production[91]. Thymic interferons do not only affect antigen presentation on individual antigen presenting cells, but also affects selection by changing the APC compartment as a whole. Here, we found that both *Ifnar1*$^{-/-}$ and *Stat1*$^{-/-}$ mice had significantly fewer hematopoietic thymic APC, the latter of which included conventional DC and B cells – both known to play a role in negative selection[9].

The dependence of IFNβ expression on AIRE was shown not only by our data of lower levels in *Aire*$^{-/-}$ and αRANKL-treated mice, but was also reflected by the overlapping dynamics of AIRE and IFNβ expression in early life. We found that the ratio of AIRE^{+} mTEC to other thymic populations changes rapidly in early life, with the highest proportion of AIRE^{+} mTEC occurring at around 3 weeks of age. This ratio and, in a 3-D sense, the larger physical network of mTEC in the thymic medulla likely informs thymocyte development: an increased AIRE^{+} mTEC to thymocyte ratio allows for increased physical interactions between the two

populations. The IFNβ production by mTEC, then, exposes a higher proportion of thymic cells, both thymocytes and other APC, to IFN-I.

In summary, we showed that tonic IFNβ is produced by thymic mTEC^{hi} in an AIRE dependent fashion. Both AIRE and IFNβ expression peak at around 3 weeks of age, before dropping swiftly and plateauing. In addition, most thymic APC respond to IFN-I, and interferon signaling in the influences thymic APC composition.

3.4 Materials and Methods

3.4.1 Mice

C57BL/6NCrl (C57BL/6) were purchased from the National Cancer Institute. B6.129S2-*Ifnar1*^{tm1Agt}/Mmjax (*Ifnar1*$^{-/-}$), B6.129S(Cg)-*Stat1*^{tm1Dlv}/J (*Stat1*$^{-/-}$), and B6.129S2-*Aire*$^{tm1.1Doi}$/J (*Aire*$^{-/-}$) were obtained from Jackson Laboratories. B6.129P2-*Ifnb1*^{tm1Acgp} mice (*Ifnb1*$^{-/-}$) were kindly provided by E. Fish and have been described previously[92]. IFN$\beta^{tdTomato}$ (IFN$\beta^{KI/KI}$) were kindly provided by D. B. Stetson (University of Washington). B6.Cg-*Mx1*$^{tm1.1Agsa}$/J (Mx1^{GFP}) were kindly provided by A. García-Sastre (Icahn School of Medicine at Mount Sinai) and have been described previously[84]. IFN-$\beta^{+/\Delta\beta\text{-luc}}$ ($\Delta\beta$-luc) were kindly provided by S. David (University of Minnesota) and have been described previously[29]. Unless noted, mice were used at approximately 5 weeks of age. For AIRE depletion, mice were injected i.p. with a single dose of 100μg of anti-RANKL (BioXCell; clone IK22/5) or isotype control (BioXCell; 2A3). Animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

3.4.2 Cell isolation

Single cell suspensions of thymocytes were isolated by mashing and filtering thymi through 70μM cell strainers (Falcon), or digestion with collagenase D (Roche, 1mg/mL) at 37°C for 30 minutes before mashing and filtering thymi through 70μM cell strainers for T cell and CD45^{+} thymic APC. Thymic epithelial

cells were isolated using Liberase TH (Roche) digestion as previously described[93]. TECs were enriched by panning (also described[93]) prior to cell sorting, but not for direct flow cytometric analysis. Cells were sorted on a FACSAria (Becton Dickinson) and populations were reliably >90% of the target population.

3.4.3 Flow cytometry

Single cell suspensions were incubated with Fc block (Tonbo) for 15 minutes at 4°C before staining with surface antibodies and viability dye for 30 minutes at 4°C or 1 hour at room temperature. For AIRE staining, cells were fixed/permeabilized using the FoxP3/transcription factor staining buffer set from either eBioscience or Tonbo following the manufacturer's protocol. AIRE staining was done at 30 minutes at room temperature. Antibodies from BioLegend were: CD4 (RM4-5), CD11c (N418), CD19 (6D5), CD25 (PC61), CD45 (30-F11), CD45.2 (104), B220 (RA3-6B2), CD64 (X54-5/7.1), CD80 (16-10A1), CD90.2 (30-H12), CX3CR1 (SA011F11), F4/80 (BM8), I-Ab (AF6-120.1), Ly-51 (6C3), Ly6A/E (D7), Ly6G (1A8), SIRPα (P84), and XCR1 (ZET). Antibodies from BD Biosciences were: CD4 (GK1.5), CD8α (53-6.7), CD69 (H1.2F3), Siglec F (E50-2440), and TCRβ (H57-597). Antibodies from eBioscience were: AIRE (5H12) , EpCAM (G8.8), F4/80 (BM8), Ly6C (HK1.4), and MHC Class II (I-A/I-E, clone M5/114.15.2). CD11b (M1/70) was purchased from Tonbo. FoxP3 (FJK-16s) was purchased from Thermo Fisher. UEA-I was purchased from Vector Laboratories.

Samples were acquired on a BD LSRFortessa, and data were analyzed with FlowJo 10.

3.4.4 Luciferase assays

For *ex vivo* luciferase measurement of populations from Δβ-luc-mice, MACS separation columns were used (Miltenyi Biotech): depletion with CD45 microbeads was followed by CD80 enrichment (labeling with PE conjugated CD80, anti-PE microbeads). Samples were treated with cell culture lysis buffer (Promega), and luciferase activity was measured in a luminometer (Berthold) using the luciferase assay system following the manufacturer's protocol (Promega).

For *in vivo* imaging, mice were injected i.p. with D-luciferin in PBS (Goldbio), anesthetized using Isoflurane (Piramal) and analyzed using a Xenogen IVIS 100 imaging system (Caliper, a PerkinElmer company). Photon flux was quantified using Living Image (Caliper).

3.4.5 Quantitative RT-PCR (qPCR)

RNA from sorted cells was extracted using an RNeasy mini kit (Qiagen). cDNA was produced using SuperScript III First Strand Synthesis SuperMix (Invitrogen). FastStart Universal SYBR Green Master (Roche) and an ABI PRISM 7900HT sequence detection system (Applied Bioscience) were used for amplification and detection. *Gapdh* was used for normalization of samples. Primers for *Ifnb1* were

forward: CCC TAT GGA GAT GAC GGA GA, reverse: CTG TCT GCT GGT GGA GTT CA.

3.4.6 Statistical analysis

Data were analyzed using GraphPad Prism software version 8.0. For comparison of two data sets, two-tailed, unpaired *t* tests were performed. For comparisons of three or more data sets, 1-way ANOVA with multiple comparisons was used for data analysis and calculation of *p* values. *P* values ≤ 0.05 were considered significant. Numbers of experimental replicates and additional details are explained in each figure legend.

3.5 Contributions

STL, OCS, and KAH designed the experiments. STL and OCS performed the experiments. STL and OCS analyzed the data. STL wrote the manuscript with input from KAH.

Chapter 4

Conclusions

The development of a functional but self-tolerant T cell repertoire is dependent on selection processes that happen in the thymus[5]. Selection is mediated by thymic antigen presenting cells, as the T cell receptors on the developing thymocytes engage with self-peptide:MHC presented by APC. Negative selection removes strongly self-reactive T cells from the conventional T cell repertoire. The APC that provide the signals that determine thymocyte fate are found throughout the thymus, and each subset has distinct localization and surface molecule expression[9]. The aim of this body of work was to gain a better understanding of how these thymic APC coordinate selection, particularly the selection of IELp and the production of type I interferon in the thymus.

To better understand selection of IELp, we made use of bone marrow chimeras with WT and β2m deficient bone marrow. We found that hematopoietic APC were required for IELp selection, rather than radio-resistant stromal cells like cTEC and mTEC. Further, CD80 and CD86 co-stimulation restrained the Type A IELp fate, so we hypothesized that the selecting APC would be $MHCI^{+}$ but lack expression of CD80/CD86. While we found several subsets of thymic APC with those characteristics, selective depletion of MHCI on individual APC subsets or depletion of the subset itself did not lead to decreased IELp selection. Rather, there was functional redundancy amongst the hematopoietic APC, and only broad defects in MHCI expression led to fewer Type A IELp. Specifically, we did not see an effect in Type A IELp numbers in CD11c Cre-$\beta2m^{fl/fl}$ or Mb1 Cre-$\beta2m^{fl/fl}$, but did see a significant reduction of precursors in CD11c Cre x Mb1 Cre-

$\beta 2m^{fl/fl}$ mice. In some ways, this was relatively surprising, as it indicated that MHCI expression on B cells was capable of supporting Type A IELp selection in the CD11c Cre-$\beta 2m^{fl/fl}$ strain. B cells, while relatively low in CD80/CD86 expression compared to cDC, are found in the thymic medulla and cortico-medullary junction[9]. Type A IELp, however, are generally cortical and are found throughout the cortex – not just clustered around the border[36]. This suggests that Type A IELp may go through the thymic medulla (or at least the cortico-medullary junction) during development, but receive signals that attract them back into the thymic cortex before emigrating from the thymus. Unlike Type A IELp, Type B IELp are generally medullary, but there is no immediate relationship between the two populations as precursor-product during development[36]. Rather, Type A and Type B are likely to be parallel developmental pathways. It is still unclear, however, if the APC that select for Type A IELp have other defining characteristics that make them 'diverting' APC other than the lack of costimulatory molecules. Further, while there is currently no tool to distinguish $CD8\alpha\alpha^+$ IEL that derive from Type A or Type B IELp, due to the differences in the precursor populations, it seems likely that they play different roles at the gut barrier surface.

To better understand the role of tonic type I interferon in the thymus, we first sought to determine the cellular source. Aligning with previous work, we found that $mTEC^{hi}$ produced IFN-I at the steady state[29, 72]. Like the name suggests, $mTEC^{hi}$ express high levels of costimulatory molecules and MHC class II[76],

positioning the thymic interferon in the environmental context of interactions that can lead to negative selection of thymocytes. Further, like the expression of tissue restricted antigens, IFNβ production in the thymus was dependent on AIRE[86]. This stringent dependence on AIRE for IFNβ production was also reflected by the dynamic peak of both early in life. This peak occurred at around 3 weeks of age and was followed by a prompt decline; intriguingly, this was independent of overall thymic size, as thymic cellularity does not follow this rapid timeline. While it has been previously appreciated that TEC populations are particularly dynamic during early life[82], this is the first time that AIRE$^+$ mTEC have been followed specifically. Using a RANKL blockade, we also showed that IFNβ expression was sensitive to RANK-RANKL signaling in the thymus, through its effects on AIRE$^+$ mTEC. The need for RANK-RANKL signaling for the differentiation of AIRE+ mTEC is well-described[78], and there is interest in repurposing RANKL blockade for cancer immunotherapy. RANKL blockade (denosumab) is currently used for bone and breast cancer due to its role in osteoclastogenesis and mammary stem cell expansion[94]. However, the reasoning behind the repurposing of RANKL blockade for other cancer types is due to its effects on AIRE$^+$ mTEC: the depletion of AIRE$^+$ mTEC will likely lead to production of more anti-tumor T cells[95]. Our work suggests that an immunotherapy that targets AIRE$^+$ mTEC would be most effective early in life, perhaps in pediatric cancer. It is possible, however, that the dynamics of the stromal compartment in mice does not represent the human thymus, and more work will need to be done.

As for the role of thymic IFN-I in tolerance, we found that most cells in the thymus respond to IFN-I, including antigen presenting cells and thymocytes. While producing IFNβ, mTEC^{hi} responded only weakly to type I interferon at the steady state. Other APC populations such as pDC, SIRPα^+ DC, and Ly6C$^+$ monocytes responded robustly, indicating that there are distinct interferon responses by APC. Further, these responses were both deeper and broader in *Stat1*$^{-/-}$ mice, indicating that other interferons, such as type II and type III, may play a role in shaping the APC compartment as well. Here, we have shown that thymic interferon changes the hematopoietic APC in terms of number. This can affect T cell tolerance by altering the likelihood of a specific of interaction between a developing thymocytes and an APC subset. For example, a thymocyte that would have undergone clonal deletion after a strong interaction with an self-peptide:MHC on an XCR1$^+$ DC no longer has that interaction due to fewer XCR1$^+$ DC in Stat1-deficient mice.

Thymic interferon may not only affect the quantity of interactions, but the quality of those interactions as well. During viral infections, dendritic cells respond to type I interferon by upregulating MHC and costimulatory molecules on their cell surface[96]. Although not explored here, it seems likely that the response to thymic interferon is similar, causing the upregulation of genes affecting antigen processing and presentation by APC subsets. Further, thymic interferon could affect not only *how* peptides are presented, but *what* peptides are presented. In support of this, others have shown that during a viral infection, the peptidome

expressed on MHCI does indeed change[32]. The qualitative impact of tonic interferon on thymic APC and the resulting changes in the T cell repertoire will be the work of future studies.

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